CONNECTIVE TISSUES

Transactions of the First Conference, April 24-25, 1950
New York, N. Y.

Edited by
CHARLES RAGAN
DEPARTMENT OF MEDICINE, COLLEGE OF PHYSICIANS AND SURGEONS
COLUMBIA UNIVERSITY

JOSIAH MACY, JR. FOUNDATION
565 PARK AVENUE, NEW YORK 21, N. Y.

Price $3.25

•



Printed in the United States of America
by Progress Associates, Inc , Caldwell, N J

PARTICIPANTS

First Conference on Connective Tissues
April 24-25, 1950

MEMBERS

W PAUL HOLBROOK, *Chairman*
Tucson Arizona

CHARLES RAGAN, *Secretary*
Department of Medicine, College of Physicians and Surgeons, Columbia University
New York, N Y

DANIEL MURRAY ANGEVINE
Department of Pathology, University of Wisconsin Medical School
Madison, Wis

WALTER BAUER*
Department of Medicine, Harvard Medical School
Boston, Mass

GRANVILLE A BENNETT
Department of Pathology, University of Illinois College of Medicine
Chicago, Ill

EDWARD W DEMPSEY
Department of Anatomy, Harvard Medical School
Boston Mass

PAUL GYORGY
Nutritional Service, University of Pennsylvania School of Medicine
Philadelphia, Pa

MOLLY R HARROWER
Psychological Consultant to Air Surgeon's Office
New York, N Y

KARL MEYER
Department of Medicine College of Physicians and Surgeons, Columbia University
New York, N Y

COLIN M MacLEOD
Department of Microbiology New York University College of Medicine
New York N Y

I ARTHUR MIRSKY
The May Institute for Medical Research, The Jewish Hospital
Cincinnati Ohio

ALBERT SZENT-GYORGYI
School of Mathematics, Institute for Advanced Study
Princeton, N J

JANET TRAVELL
Department of Clinical Pharmacology, Cornell University Medical College
New York, N Y.

B W. ZWEIFACH
Department of Medicine, Cornell University Medical College
New York, N Y.

GUESTS

T DUCKETT JONES
Helen Hay Whitney Foundation
New York, N Y

JOHN R MOTE
Armour Research Foundation, Armour Laboratories
Chicago, Ill

GERTRUDE E PERLMANN
Department of Protein Chemistry, Rockefeller Institute for Medical Research
New York, N Y.

JOSIAH MACY, JR. FOUNDATION

FRANK FREMONT-SMITH, Medical Director
JANET FREED, Assistant for the Conference Program

TABLE OF CONTENTS

JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT-SMITH
Medical Director

I AM delighted to welcome you to the first meeting of the Conference on Connective Tissues and to give you as briefly as possible an outline of what we hope to accomplish by these conferences.

With the accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece, it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress.

Nowhere in science is the need for "combined operations" more evident than in medicine. Today, to be effective, medical research and practice must embrace data from all of the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other, for advances in one field are frequently [illegible] thus [illegible] not yet made adequate provision for channels of interdisciplinary communication.

The Foundation is interested in furthering knowledge about connective tissues and is also interested in the broad aspects of the problems of communication and integration which are important for the advancement of the whole of science. It is our belief that scientific communications at scientific meetings and in the journals have been forced into a narrow mold in which logical sequence leading to inevitable conclusions is substituted for the much more flexible and often unpredictable processes by which scientific inquiry and the advance of knowledge actually take place. All of the creative, the really exciting and interesting, factors which are the soul and heart of science tend to be excluded today. This unfortunately discourages individuals who have a creative and artistic turn of mind from entering the scientific field

and creates in the minds of students and of the public a profound misunderstanding of the nature and processes of science.

The Foundation's experience with the many research projects coming before it has led to the conviction that one of the greatest needs today is the reintegration of science now artificially fragmented by the isolation of the several scientific disciplines and specialties. Our Conference Program hopes to encourage this reintegration and to give in the published transactions of our conferences a clearer reflection of what takes place in the laboratory and what goes on in the minds of investigators than now appears in scientific literature.

Thirteen groups are now functioning under the Conference Program and cover the following topics: Adrenal Cortex, Aging, Biological Antioxidants, Blood Clotting, Blood Pressure, Connective Tissues, Consciousness, Cybernetics, Infancy and Childhood, Liver Injury, Metabolic Interrelations, Nerve Impulse, and Renal Function. The printed transactions are made available at cost.

Each group will hold annual two-day meetings for a period of five years. It is our belief that only through continued association in an atmosphere of friendliness and mutual confidence can effective communication (exchange of ideas, data, methods, and plans) across the barriers of the professions and specialties be promoted. As a result of these meetings we have seen plans and ideas modified, conclusions more clearly specified or placed in a broader perspective, and spontaneous collaboration take place between investigators working in different departments or in different universities.

As a nucleus, fifteen scientists comprise the original group of members for any conference. These are selected by the Chairman of the Conference in consultation with the Foundation. Every effort is made to include representatives from all pertinent disciplines. From time to time new members are added by the group to fill gaps in viewpoint or technique. A limited number of guests are invited to attend each meeting, but, for the purpose of promoting full participation of all members and guests, attendance at any meeting is limited to twenty-five. It is inevitable that in no topic can we possibly include even more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include.

A point which I should like to stress before closing is that between the disciplines there are real difficulties in communication—partly emotional and partly semantic. Emotionally some of us accept only data coming from those methods or disciplines

with which we are familiar. It is important that we do justice to the validity of data and methods from other disciplines. On the semantic level, the physical and biological sciences have little difficulty; the medical, psychiatric, and social sciences can understand each other fairly well; but to bridge the gap from the physical and biological sciences to the psychological and social is very difficult. However, in the study of man all of the sciences must meet. In medicine, which must be equally concerned with the psychological and social as with the biological and physical, there is the greatest opportunity, as well as necessity, for mutual understanding among representatives of all the sciences. I believe *that the hope for a unification of science lies in the development of a Science of Man* in which medicine must play a central role.

In closing, I want to say that the Conference Program is an experiment and that you are part of that experiment. We hope that at these conferences you will feel the freedom inherent in the scientific method and will help us to improve our conference procedure.

INTRODUCTORY REMARKS

W. PAUL HOLBROOK
Chairman

INASMUCH AS this is an informal conference, your Chairman has no prepared remarks. The more I have thought of the title, "Conference on *Diseases of Connective Tissue*," the more I have been confused regarding a place to begin.* I would like, therefore, to suggest that we at the moment concern ourselves less with diseases of connective tissues, and more with connective tissue itself. I hope that we will feel as free to say and discuss what we do not know and what we ought to find out as we are to talk about a very few of the things that we do know in this field. One more thought — it is much more important to this Conference that we discuss what we are thinking rather than those things which may have already been proved. Ideas, I am sure, will be fully as valuable as facts. I hope we can have complete informality, interruptions, and discussions at any time during the Conference. This Conference offers us a rather unique opportunity to sit down leisurely and calmly and talk intimately with a group of people representing widely diversified disciplines. It is an occasion to which I have looked forward with a great deal of pleasure. I believe we are ready to start our first topic and the opening discussion with Dr. Angevine telling us about the structure and function of normal connective tissue.

*Name of Conference changed at the meeting to "Conference on Connective Tissues."

STRUCTURE AND FUNCTION OF NORMAL CONNECTIVE TISSUE*†

D. MURRAY ANGEVINE
Department of Pathology
University of Wisconsin Medical School

I SHOULD like to thank Dr. Fremont-Smith for his remarks, because they have answered many questions that have arisen since Dr. Holbrook invited me to participate in this Conference.

Not being an anatomist and also because there is one in this group, I was rather hesitant to discuss this subject. In medical schools we always teach the normal and feel that familiarity with it must be acquired before one can understand or recognize the abnormal, although I am not certain that one can really know the normal until one knows something of the abnormal. For this reason I have felt justified in undertaking this presentation. To further illustrate this, perhaps no one would know what a beautiful woman was unless they were familiar with the opposite. Here, of course, it is difficult to say which is the normal and which is the abnormal.

In starting a discussion of connective tissue I feel very much like an explorer being launched into the stratosphere on a rocket. Probably we know a little about the topography of the sun and moon and perhaps some of the major constellations. The rest of the universe is about as unexplored as, and may be compared with, connective tissue or at least the intracellular connective tissue. What goes on there is almost anybody's guess at the present time, and anything I may say about it is wide open for discussion.

Most histologists are in essential agreement on the classification and anatomical structure of the connective tissues, but they are not in agreement as to embryogenesis, or development, or as to function. *For a good many years scientists have been trying to* indicate that there is some significant role in the physiology of connective tissue but they have not made very much headway

*Dr. C. H. Altshuler was the principal investigator in most of the work to be reported.

†These investigations have received financial support from the U. S. Public Health Service, Wisconsin Alumni Research Foundation, Thomas F. Brittingham and the Best G. Heath Fund.

I think that the initial recent interest in the subject began following the demonstration of certain specific lesions of disease in connective tissue, especially in rheumatic fever.

I hope anyone will interrupt as I go along.

Fremont-Smith: Just to reemphasize what you said about learning the normal from the abnormal, I am sure that we only really can learn the normal from the abnormal, and that the process of going at the normal first is an error. We should go at it in the reverse way, which we can do clinically. It is from the deviations brought to our attention that we get at the nondeviants. I just want to illustrate that we do interrupt here.

Angevine: That becomes more apparent to us as we teach pathology to second year medical students. Although they have completed a course in histology, they seem totally unaware of certain structures until they observe them in relation to the abnormal.

Jones: Isn't it more the usual or the average rather than the actual normal? Anyway the variants are so wide, and function within that range so variable, that I think we have been held back in our medical knowledge on the basis of what is a normal individual in relation to any particular problem.

Meyer: Whether to go from the normal to the pathological or vice versa depends on what we are studying. At the structural level and the cellular level, we probably ought to, and we certainly can, start from the pathological and go to the nonpathological. But if we go to molecular level, including both chemistry and part of physiology, we have to start from the normal since beyond some primitive beginnings we know very little about the pathological chemistry and physiology of most fields of medicine and nothing at all about the physiology of either the pathological or the normal connective tissue.

Fremont-Smith. You will have a chance, sir. You might say that the protein molecule, perhaps denaturization, has given us approaches to the normal which we never would have obtained if we had not seen coagulation—again it is the deviant. I wonder whether the radioactive atoms, which are the unusual ones, have not taught us more about the nonradioactive atoms than we ever could have known otherwise.

Mirsky: I was fascinated to hear that "communication" was to be the topic of discussion and then to note that the first words uttered by the speaker led us immediately to that problem because he used the word "normal." I was disturbed. I did not know

whether he was about to discuss mathematics, some imaginary distribution curve or some chemical element. I was about to ask, "What do you mean by 'connective tissue,' even before you tell us what you mean by 'normal' and 'abnormal'?" Many people think they are normal because they happen to have a certain concept of normality, some mean into which they believe they fit. Only when they happen to go into some other community do they find that they do not "fit" and therefore, are abnormal. I believe that even at the molecular level we must talk only in terms of statistical probabilities rather than in terms of the ill-defined areas of "normality and abnormality." Let us be descriptive only for the time being and not try to categorize.

Fremont-Smith: Let me break in again. In editing the volume on Problems of Aging, we had difficulty defining the meaning of "normal," in trying to distinguish normal aging from the inroads of pathological processes. For example, the normal kidney, as one moves on from young life to middle life, has a series of glomeruli which die, degenerate, and disappear. This process, so far as we know, occurs in every normal kidney. Therefore, you reach the point where it is necessary to talk about the pathology because a dead glomerulus is a pathological glomerulus. That shows the difficulty in our terminology. I think if we try to be as precise as we can, and where we are not precise try to make it as clear as possible, we can move forward. I am really the one who broke this up.

Holbrook: Dr. Angevine, that puts quite a load on you for your terminology.

Angevine: Yes.

Fremont-Smith: I would go on and say what you planned to.

Angevine: Much of the recent interest in this field has developed largely because of newer techniques in science such as tissue culture and electron microscopy. These two techniques and the advances in histochemistry have aroused our interest more than anything else. Finally, the greatest impetus—and I suspect the impetus of this Conference—very well may have been initiated by the observations of the effects of cortisone and ACTH on this particular system. I believe that these techniques and these substances may open up many of the mysteries of the interstitial tissues which have challenged investigators for years.

Time is not going to permit a detailed histological description of these structures. I will cover the essential features rather briefly. You will find them described in any good treatise on histology (1, 2, 3). I was amazed, however, in comparing standard textbooks

to find many differences of opinion on this particular subject.

Connective tissue comprises a number of tissues that arise from mesoderm and are principally concerned with the formation and maintenance of bodily structure, namely, bone cartilage and connective tissue proper. My remarks will be confined to the latter, namely, connective tissue proper. This is usually divided into four types: loose connective tissue; dense connective tissue; regular connective tissue; and connective tissues with specialized function, such as fat, mucin, and so forth.

All of these tissues are composed of a homogeneous matrix or ground substance in which are incorporated certain fibers and cells, and it is only the relative preponderance of either one of these elements that determines the classification of the tissue. This is, of course, a very general statement, and since the same fundamental problems pertain to all types, I will concern myself with loose connective tissue only. This binds part of the body together and also permits of considerable movement in certain areas. It is widely distributed, principally in the subcutaneous tissues, in the submucosa of the gastrointestinal tract and subserous surfaces beneath the pericardium, the pleura and the peritoneum, also between and around vessels and nerves as well as throughout internal organs.

Embryonic connective tissue, or mesenchyme, is the tissue from which this loose connective tissue derives This is composed of very small cells with slender continuous branching processes that form an exceedingly fine network from which the various elements of mature connective tissue differentiate. Many scientists consider that loose connective tissue is an undifferentiated form of embryonic connective tissue. It certainly has many potentialities and can develop into almost any tissue. If one distends connective tissue with air or fluid, it immediately becomes evident that there are large numbers of spaces between the various fibers, and it is in this area that the ground substance exists. In addition to the ground substance there is probably tissue fluid, which may in some areas be incorporated with the ground substance and in others may be separate. The tissue fluid represents material that comes from the plasma, the water soluble proteins, metabolites, crystalloids, gases, and so forth. It may be likened somewhat to absorbent cotton with the ground substance representing the interstices between the cotton fibers. It is composed of cellular and intercellular elements, the latter containing collagenous or white fibers and elastic or yellow fibers between which there is an amorphous ground substance.

I would like to say something about the collagenous fibers, and after that discuss some of the other elements. There has been a great deal of interest in collagen diseases and collagen fibers, but I am going to point out as I go along that most of the changes in collagen are probably secondary, *although not by any means of* lesser importance. The cart may, therefore, have been placed before the horse in our understanding of these diseases. Collagenous fibers are formed of bundles of small fibrils. They run a wavy course and are very flexible and resistant to force. They are composed of an ill-defined material called collagen albuminoid. This material can be converted to gelatin by boiling, and that is what *the term means, "production of glue."*

When these fibers are exposed to weak acids or alkalies, they will swell. If placed in strong acids or alkalies they will disappear or be destroyed. X-ray diffraction studies have indicated that as near as they have been able to define them collagen fibrils are composed of long chain polypeptides. Electron microscopy has clearly demonstrated the detailed structure of these collagen fibrils *of which we were not aware. There are definite cross striations* not dissimilar to those seen in muscle. There is also a definite periodicity to these cross striations.

Fremont-Smith Periodicity in what sense?

Angevine That the striations are regularly spaced. In this connection Gross (4) has recently reported—and this is something that we have suspected for years but had always hoped someone might prove—that reticular and collagen fibers are of essentially similar origin. He has shown by electron microscopy and x-ray diffraction that reticular fibers are probably just young collagen fibrils and, although they stain somewhat differently, this is only due to the fact that they are younger, and as these reticular fibrils develop they increase in size to form collagen. This is a significant observation.

The elastic connective tissue fibers are much less numerous, are single, branch and anastomose freely, are highly refractile and elastic, and are yellow in color. If there are a sufficient number of them in any tissue they will give a yellow color to it as in certain tendons and ligaments in which they exist. They are also supposed to consist of an albuminoid called elastin, which in contradistinction to collagen is highly resistant to strong acids and alkalies, and it will also resist boiling, so it is a much more resistant structure.

MacLeod It does not swell under those circumstances?

Angevine It does not swell to the same extent as collagen.

Meyer It does not go into solu[illegible]nder the same condition

Angevine: No. It will withstand very strong acids and alkalies. *I am not certain of the exact strength. There are certain specific* stains for these fibers. Collagen stains red with van Gieson's stain and elastic fibers are colored red brown with orcein.

Much important work is now being done to define the physical and chemical characteristics of collagen. I think it also highly important that we have more accurate information on the process of fibrillogenesis; that is, how these fibrils form. The usual concept is that the collagen fibrils form directly from the processes of the fibroblasts. However, on the basis of experimental evidence, others consider the action to be more indirect, and that the fibroblasts elaborate some material into the ground substance with which it will be combined to initiate fibril formation.

There are several observations in this connection that, in my opinion, are significant. Stearns (5) at the University of Pennsylvania studied fibrillogenesis in the transparent chamber in the rabbit's ear. During this process she saw small granules accumulate in the cells that escaped into the ground substance, and these small particles rapidly formed small fibrils in the ground substance. The process was rapid; they formed within two to four hours, and sometimes after forty-eight hours they were so numerous that they would obscure the original cell These observations made about ten years ago seem to correspond with the recent observations of Gersh (6) that there is a material in the fibroblast called a glyco-protein which has a carbohydrate radical attached to it and is the precursor of the ground substance. This material is first observed in the cell and is then seen in the ground substance. Gersh believes that the fibroblasts secrete this material which forms the ground substance. He does not, however, discuss the formation of the fibrils.

Tissue culture studies (7) have supported the observations that have been noted in the rabbit's ear, and they indicate that the fibrils originate outside of the cells, probably from the substance secreted by them Tissue cultures have also shown no essential morphologic difference between reticular and collagen fibers.

Some of the earlier workers considered that fibrin had something to do with the formation of fibrils, but this is not so because fibril formation has been demonstrated in tissue cultures free from plasma; in other words, there is no possibility of fibrin being present.

Another interesting observation that has been made both on tissue culture and on the rabbit's ear is that if one exerts stress or tension on the culture or the ear by pressing on the tissue

culture or by pulling the ear, there is a mechanical stimulation to the formation of fibroblasts. They will form in greater abundance, and also the direction of the stress will determine the direction in which the fibroblasts are laid down.

Elastic fibrils have been grown in tissue culture from the heart and aorta by Bloom. They grow very sparsely and take about twelve days to appear. They also develop outside of the cells but in close connection or proximity with them.

Ragan: Are they continuous with the cell?

Angevine: No, they are not. Jordan (2) says they are, but as near as I can determine they are not. There is a great difference of opinion between standard texts, as I indicated previously.

The recent reports of Plotz and his co-workers (8), as well as the reports of Spain (9), that cortisone and hyperadrenalism interfere with the formation of granulation tissue and also with the normal callus formation or repair of bone are of the greatest interest. They suggest that the mechanism of fibrillogenesis may very well be under the control of the endocrine system. I will not say any more than that on this subject at present.

It is also well known that vitamin C is necessary for the formation of collagen fibrils (10). Elster (11) has recently reported that once the collagen fibrils form, vitamin C is not necessary to maintain them. Due to the relationship of vitamin C or possible control of it by the adrenals, the inference is again that the adrenals may be more significant in this connection than previously suspected.

We believe—and when I say "we" I mean Altshuler, who has done most of this work—that the ground substance is the area to which most attention should be devoted. In fact, most of the alterations in collagenous tissue may well be secondary to alterations in the ground substance. Whether or not this will eventually prove to be true is, of course, yet to be established.

I will now turn to the physiology of connective tissue and discuss this ground substance somewhat more in detail With the light microscope this tissue is structureless and cannot be seen However, it becomes very apparent when stained with certain [illegible]

[illegible] red color. By "metachromatically" I mean they stain a different color with toluidine blue If they stain blue with toluidine blue, they are not metachromatic, but if they stain red they are metachromatic. This altered staining is spoken of as *metachromasia.*

Angevine: No. It will withstand very strong acids and alkalies. I am not certain of the exact strength. There are certain specific stains for these fibers. Collagen stains red with van Gieson's stain and elastic fibers are colored red brown with orcein.

Much important work is now being done to define the physical and chemical characteristics of collagen. I think it also highly important that we have more accurate information on the process of fibrillogenesis; that is, how these fibrils form. The usual concept is that the collagen fibrils form directly from the processes of the fibroblasts. However, on the basis of experimental evidence, others consider the action to be more indirect, and that the fibroblasts elaborate some material into the ground substance with which it will be combined to initiate fibril formation.

There are several observations in this connection that, in my opinion, are significant. Stearns (5) at the University of Pennsylvania studied fibrillogenesis in the transparent chamber in the rabbit's ear. During this process she saw small granules accumulate in the cells that escaped into the ground substance, and these small particles rapidly formed small fibrils in the ground substance. The process was rapid; they formed within two to four hours, and sometimes after forty-eight hours they were so numerous that they would obscure the original cell. These observations made about ten years ago seem to correspond with the recent observations of Gersh (6) that there is a material in the fibroblast called a glyco-protein which has a carbohydrate radical attached to it and is the precursor of the ground substance. This material is first observed in the cell and is then seen in the ground substance. Gersh believes that the fibroblasts secrete this material which forms the ground substance. He does not, however, discuss the formation of the fibrils.

Tissue culture studies (7) have supported the observations that have been noted in the rabbit's ear, and they indicate that the fibrils originate outside of the cells, probably from the substance secreted by them. Tissue cultures have also shown no essential morphologic difference between reticular and collagen fibers.

Some of the earlier workers considered that fibrin had something to do with the formation of fibrils, but this is not so because fibril formation has been demonstrated in tissue cultures free from plasma; in other words, there is no possibility of fibrin being present.

Another interesting observation that has been made both on tissue culture and on the rabbit's ear is that if one exerts stress or tension on the culture or the ear by pressing on the tissue

culture or by pulling the ear, there is a mechanical stimulation to the formation of fibroblasts. They will form in greater abundance, and also the direction of the stress will determine the direction in which the fibroblasts are laid down

Elastic fibrils have been grown in tissue culture from the heart and aorta by Bloom. They grow very sparsely and take about twelve days to appear. They also develop outside of the cells but in close connection or proximity with them.

Ragan: Are they continuous with the cell?

Angevine: No, they are not. Jordan (2) says they are, but as near as I can determine they are not. There is a great difference of opinion between standard texts, as I indicated previously

The recent reports of Plotz and his co-workers (8), as well as the reports of Spain (9), that cortisone and hyperadrenalism interfere with the formation of granulation tissue and also with the normal callus formation or repair of bone are of the greatest interest. They suggest that the mechanism of fibrillogenesis may very well be under the control of the endocrine system I will not say any more than that on this subject at present.

It is also well known that vitamin C is necessary for the formation of collagen fibrils (10). Elster (11) has recently reported that once the collagen fibrils form, vitamin C is not necessary to maintain them Due to the relationship of vitamin C or possible control of it by the adrenals, the inference is again that the adrenals may be more significant in this connection than previously suspected

We believe—and when I say "we" I mean Altshuler, who has done most of this work—that the ground substance is the area to which most attention should be devoted In fact, most of the alterations in collagenous tissue may well be secondary to alterations in the ground substance. Whether or not this will eventually prove to be true is, of course, yet to be established.

I will now turn to the physiology of connective tissue and discuss this ground substance somewhat more in detail. With the light microscope this tissue is structureless and cannot be seen. However, it becomes very apparent when stained with certain dyes such as toluidine blue. Because it contains mucopolysaccharides, of which hyaluronic acid, chondroitin and mucoitin sulfuric acid are examples, it stains metachromatically, namely a purple red color. By "metachromatically" I mean they stain a different color with toluidine blue If they stain blue with toluidine blue, they are not metachromatic, but if they stain red they are metachromatic. This altered staining is spoken of as

In connective tissue the polysaccharide stains a purple red in color. Dr. Meyer has shown that any large molecular substance with a free acid radical may induce metachromasia. This property is not confined entirely to mucopolysaccharides, however, so that these stains are not entirely specific.

To go a bit further, and this is more our postulation—Dr. Fremont-Smith said that you are interested in what we *are* thinking about rather than what we *have been* thinking of in the past. I would like to refer briefly to the hematoparenchymal barrier; in other words, when any material passes from the blood stream to a cell it must go through several structures. It must go through the capillary membrane. It must also go through interstitial tissue or fibrous connective tissue composed of ground substance, and must pass through the cell membrane. To re-enter the circulation it must traverse the same structures in reverse order. These structures collectively are spoken of as the hematoparenchymal barrier. When there is an accumulation of any cell-free fluid in this area it is spoken of by some as "serous inflammation" (12). This is not a particularly good term It was first described by Rössle (13) and elaborated by Eppinger (14), and various people from time to time have attempted to explain the mechanism. Some have devoted all of their attention to the capillary membrane; others have devoted all of their attention to the ground substance; and others to the cell wall. It has been shown that a variety of injurious agents at such a site will produce many alterations. Some of the agents that have been used to damage this area have been allyl formate, thyroxin, chronic fatigue, or anoxia. If one damages tissue with any of these agents—and a good many more might be listed—the injury is always associated with an excessive accumulation of mucopolysaccharides in the interstitial tissue This protein-rich fluid separates the capillaries from the parenchyme and impairs the nutrition and excretory ability of the parenchymal cells. This, in turn, leads to depressed cellular respiration and relatively *increased glycolysis. It has been our experience that in many* instances where such conditions obtain and are maintained for some time, there is an accumulation of materials which yield a metachromatic reaction to toluidine blue.

So much for the ground substance. This brings us to a word about the various cellular elements of which the most important, I believe, are the fibroblasts and the macrophages. The fibroblast in connective tissue, as I have indicated, plays a role in the formation of fibrils. It also contains large bodies in the cytoplasm that some have called mitochondria; others speak of them as fibrochondria. They have not been extensively studied. If they are

mitochondria, it may indicate that they have some relationship to enzyme activity Harman has shown in kidney and liver that enzyme activity and intactness of mitochondria are closely related (15).

The fibroblast, in addition, plays an important role in water retention in tissues. It has been noted that these cells show wide fluctuation in size under different conditions of hydration, and this property of taking up water from surrounding media will occasionally lead to local edema.

The cells of the macrophage system deserve a paper by themselves, but I will merely indicate that they form a protective and scavenging system, and that they occupy most of the tissues in the body, in addition to these there are lymphoid cells, mast cells, eosinophils, plasma cells, fat and pigment cells, all of which have their own functions. These brief remarks should indicate that connective tissue, rather than being an inert structure with a passive function, is a very active tissue and probably has many significant functions. In addition to support and packing properties, it may be considered as a generalized tissue capable of giving rise to more specialized elements under certain circumstances It may form a panniculus adiposus, which allows for storage of fats and provides a covering which helps conserve body heat It plays a significant role in tissue repair by the deposition of collagenous fibers to form scar then connective tissue containing tissue fluid which is an essential medium through which cellular elements may function in relation to blood and lymph.

In conclusion, I would like to indicate some of the areas of exploration that I feel this brief survey leads us to: 1) Further cytochemical studies on the fibroblast are indicated, with special reference to the function of fibroblasts. 2) There should be an exploration of the enzyme systems in relation to connective tissue. Most of the studies of enzymes, as you know, are done on parenchymal cells, especially on the liver, kidney, and secretory cells. 3) Further studies are indicated to define more precisely the process of fibrillogenesis. 4) Define more accurately the function of the ground substance. 5) We also are concerned in the mechanism whereby these various processes are controlled 6) Studies of normal physiological processes, especially repair and wound healing, will provide further valuable information in this field

Holbrook: Thank you very much, Dr Angevine, for bearing with our interruptions.

Dr. Dempsey, have you anything you would like to add to this discussion at the moment?

Dempsey: I haven't anything in an organized form. There are, however, two or three comments I might make. It might be worth while to belabor this point of normal versus abnormal a little more. I am not sure we are ever going to agree on just what these words do mean, because as ideas become crystallized more and more sharply we will have to invent new words to get away from the unwanted connotations of the old ones. Nevertheless, it is easy to demonstrate that a normal individual may have abnormal tissues, cells or other structures within his body; and conversely that an abnormal individual may have certain normal structures. Thus, for normal or abnormal, we must define the level of organization. Shall we consider the whole individual as normal or abnormal, or shall we restrict our attention to the component parts of the individual as normal or abnormal? This distinction is perhaps not too farfetched because one of the only ways we really have of establishing what is normal or abnormal is to take the tissues of a sick individual, who is by definition abnormal. Therefore, we define his tissues as abnormal, whereas they may or may not be. Then the issue becomes even more complicated when we try to understand the different appearances of what must be perfectly normal tissues. Growing tissue, for example, is healthy, yet its appearance is very different from the appearance of the same tissue after growth has been attained. Yet both are "healthy" and presumably "normal." Likewise, the cells that Dr. Angevine mentioned, the mesenchymal cells, are detectably different from the fibroblasts to which they give rise. They are also the progenitors of fat cells, macrophages and other cell types of connective tissue. Now all of these are normal; they seem to represent different appearances at a time when the cells are doing different things. So normal has to be defined against the basis of what the cell is doing, what its present physiological situation is, what its physiological purpose is—if you will permit the use of that word.

Thus we come down to the question of whether any of the alterations ordinarily recognized as pathological really represent inherently pathological processes, or only the normal response to an altered physiological situation.

Fremont-Smith: If one adds the phrase "with respect to" each time one uses the word "normal" or "pathological" or any of these generalizations it helps enormously.

Dempsey: If he makes his description precise.

Holbrook: I would like to ask you, Dr. Angevine, if there is any variant in connective tissue structures which varies with age, and

might give us a lead toward certain abnormal states influenced by age?

Angevine. Yes, it has been shown that there is a definite alteration of these particular fibers of collagen during growth. As age progresses, there is less ground substance present. That seems to be confirmed by several workers I believe Gersh agrees with this. The collagen fibers become more compact and are less loosely arranged. I know of no definite alteration in the collagen fibrils themselves. I know there are certainly no alterations in the amount of collagen Harman (16), in our department, has been working on aging in the human heart. He took forty hearts presumed to be normal. At least they had no demonstrable coronary vascular disease, and the circulation was considered adequate, and there was nothing to indicate disease of the myocardium during life. He took portions from each chamber of the heart and determined the amount of elastin and the amount of collagen in all chambers. We expected an alteration as age increased, but to our amazement there was no difference in the quantity of collagen or elastin in hearts from young or old adults I am not saying anything about the myocardium, but the structural supporting framework of that heart was not altered quantitatively That was surprising to us.

Fremont-Smith May I add that in Cowdry's book on aging, where every organ system was referred to, the same conclusion was reached—that each organ was not the site of serious disease process itself. If the patient had died from cerebral accident, or from an accident in some other organ system, that system being surveyed, whether eye, ear, brain, or muscle, showed no evidence whatsoever of not being able to go on indefinitely to perform its function. The only conclusion I could draw from reading that book was that people did not die and that did not seem to be a logical conclusion! *There is a problem before us which we cannot solve at the moment.* What do we mean by "aging"? One sometimes attempts to ask whether it is the interrelating factors, *the homeostatic functions themselves, which break down; that* you reach the point perhaps that normal aging would lead to, the "one horse shay" situation where suddenly everything goes to pieces at once. Whether anybody has ever seen normal senescence of any organ system is a question.

Mirsky You mean morphologically or in terms of functional capacity?

Fremont-Smith No particular organ system; for example, an individual of ninety-four with an organ system which was not the

cause of death, but could have carried on for forty or fifty years. I did not say "no evidence of aging" but "no evidence that *the end* of functional capacity had even been approached."

Meyer: I would like to come back to the work about the relation of collagen and elastin to development and aging. The ground substances may have a lot more to do with these processes than the fibrous elements. That brings up the question of the main function of connective tissue which Dr. Angevine mentioned in the beginning, namely, that its main function is the maintenance of form and the architectural relationship in the body. Is the main function of the connective tissue a purely passive one, or are we dealing there with a highly integrated system in a functional sense, between the connective tissue and the structures it "supports," the epidermal structures, the glandular tissues and so on? It should be assumed that there are such interrelating functions which are disturbed in aging as well as in some pathological processes. By the way, the only experimental work on such an integrated physiological process between an epithelial structure and connective tissue that I know of is that of Friedenwald on the secretion of ocular fluid by the ciliary body. According to Friedenwald there is a potential difference between stroma and ciliary epithelium. This potential generates a current while carrying water from stroma to epithelium and out as ocular fluid. While some of the details of the interpretations may be questionable, the main idea of active chemical cooperation between epithelium and stroma seems to me highly probable for all epithelial as well as endothelial structures.

Mirsky: Would you say the recent work on the relation of fat metabolism to glycogen synthesis in the fat cell is also indicative of a specific function of connective tissue?

Meyer: I don't know enough about the synthesis of fat, where it takes place, and so forth. There is apparently a big gap there.

Holbrook: Is it agreed that the structure of connective tissue is not significantly altered by age alone to be indicative of a disease process that might be present at such an age?

Angevine: I would say yes, so far as the individual fibrils are concerned. Gersh (6) confused me a bit by speaking of loose connective tissue and basement membrane, meaning by "basement membrane" a certain condensation of this tissue in some areas, especially around the kidney tubules, and so on. I believe he has indicated that initially there is loose connective tissue and as one becomes older the fibers that are widely dispersed come together in a more compact manner. I infer that this occurs because there is

less ground substance between them. If one looks at a section of aorta from a child, the fibers are definitely looser in arrangement than in older individuals.

Dempsey: I recently heard Gross read a paper on the size of collagen fibers in rats of different ages. What he did was to shake the connective tissues apart and examine them with the electron microscope. He then measured the width of the fibers and made a distribution plot of the size of the fibers. He showed, as you might suspect, that early in life the fibers were all small, and that they seem to coalesce, or at least to increase in diameter as the age of the rat increases.

It seems to me that this observation fits in with the statement you just made to the effect that the aged individual seems to have a denser and more solid collagenous tissue framework than does the younger one. However, I am not so sure that a removal of ground substance between the fibers is the cause of this solidification. It could be that the fibers themselves may enlarge by coalescence.

Bennett: The elastic qualities of this type of tissue seem to diminish somewhat proportionately to its condensation. In young subjects, the dermal structures are elastic and when mechanically pulled up and released, they quickly snap back into their original position Histologically these tissues are loosely textured. In older subjects when the same dermal areas are pinched together and released, *they slowly return to their normal position.* On [illegible] scopic examination such tissues [illegible]

We can [illegible]

exampl [illegible] elasticity which seems t [illegible] morphologic appearances that imply condensation of tissue components. Whether or not this alteration in appearance is related to diminution in ground substance is not known to me. I would be interested in hearing what Dr. Angevine has to say on this point

Angevine: I don't know either, Dr. Bennett One of the best illustrations—it is not mine—of how tissue may age is the example of the agar plate. One makes two fresh agar plates and removes the cover from one; it dries and loses its gel properties, whereas the other one maintains them. I imagine in aging there could be much interference with passage of substances across this ground substance leading to an accumulation of these materials. Whether it is an alteration or loss of ground substance would be difficult to determine I don't know how it could be Do you, Dr. Meyer?

Meyer: There is nothing known about qualitative changes in

the ground substance with age. In embryonal tissue, that is, umbilical cord, we find not only very much larger quantities of mucopolysaccharides than in adult connective tissue, but we find the complete absence of one component, the chondroitin sulfate B, which we find in every other connective tissue we have examined.

Fremont-Smith: You know about its presence in other embryonic tissue?

Meyer. We have not examined other embryonic tissues.

Fremont-Smith: You have not had a chance to investigate yet?

Meyer: The absence of this mucopolysaccharide seems significant to us in view of the absence of the basement membrane reported in Gersh's paper and the increase in thickness of this membrane on aging, not only in skin but also in blood vessels. Gersh's finding is based on the periodic acid-Schiff's reagent staining. I wonder, Dr. Angevine, whether the thickening of the basement membrane can be explained by the collapse of the fibers or by the disappearance of ground substance? I thought the fibers are not stained by the periodic acid-Schiff stain, which is supposed to indicate glycol groups or similar structures, which form aldehyde on periodic acid oxidation. I guess this may cause quite some discussion here.

Dempsey: One of the things the periodic acid-Schiff method demonstrates beautifully is reticulum. There is, I believe, a considerable quantity of alpha-hydroxyamino acid in collagen hydrolysates, and this, rather than neighboring glycol groups, might account for the Schiff reaction.

Meyer: Alpha amino-beta hydroxy acids, you mean. This would imply that the amino groups were free and not present in a peptide bond. This would happen only in end groups, of which there could not be many in a protein. The histologists have not paid much attention to the quantitative aspects of this staining method, namely, how much protein or carbohydrate has to be present in a given volume to be visible with the Hotchkiss stain.

Holbrook: I would like to raise one question about the barrier Dr. Angevine talked about. In the barrier, other than the connective tissue cells which you discussed, are there any findings structurally which can be seen with variations in age? I am trying to find out whether structurally you can see at this site of connective tissue disease, anything that might change. Function I am not talking about; we know there are changes in function.

Angevine: Not that I know of, Dr. Holbrook.

Zweifach: Volterra has described specific changes in the so-called pericapillary sheath which develop with age and under

different pathological conditions (17). Several Russian papers have also stressed the importance of changes in the argentophilic properties of the hematoparenchymatous barrier in aging and especially in the development of hypertension (18).

Angevine: I have talked to Dr Meyer of this. Altshuler and I have approached this subject more or less system by system; having worked first with fibrinoid we are now following the alterations that take place following injury to the hematoparenchymal barrier. Such a study has been made in the lungs. We have also made such a study in relation to the blood vessels and nerves. We have observed similar alterations occurring in these areas, in the interstitial tissues of the lungs, the vascular tissue throughout the body, and also in the nerves. The subject is so diffuse that we felt by keeping our observations for a time in separate compartments it might mean more to those interested in special fields such as diseases of the lungs, the cardiovascular system, and so forth We have made no observations in relation to age.

Ragan. May I ask what is the distinction between capillary membrane and basement membrane?

Zweifach: I do not believe that we are justified in comparing the capillary membrane directly with a structure such as the basement membrane. The capillary wall is a complex structure made up in part of endothelial cells cemented together by an intercellular substance. Surrounding the endothelial membrane is another membrane, the so-called pericapillary sheath. These two structures should be considered separately since the evidence indicates that the interendothelial cell cement and the pericapillary ground substances do not have similar chemical properties. For example, extracts with hyaluronidase activity affect the pericapillary sheath, causing it to soften, whereas the cement substance binding the endothelial cells together is not affected by such enzymes. Calcium salts likewise affect the two structures differently. We therefore cannot assume that the cement substances which bind cells together and those which form basement membranes will be similarly affected under comparable pathological conditions.

Angevine I think that is true. I have felt that basement membrane being applied to this subject has been a bit confusing because my conception—and some will disagree with me on this—of a basement membrane was a limiting membrane that limited epithelial cells or occurred in certain structures as in the Descemet's membrane of the eye. Gersh has assumed it to be areas in which there is a condensation of fibers, in which there

is a condensation of membrane, and I would not consider that the surrounding tissue about a small blood vessel would be considered as basement membrane. I would call that perivascular adventitial tissue, just to keep our terminology straight. I have no quarrel with his observations, since I introduced the subject by intimating that connective tissue can be divided into several types, loose and dense. Dense connective tissue is what I believe Gersh is talking about when he speaks of membranes beneath the skin. If one keeps that conception it may make discussion a little easier.

The same thing applies to the kidney tubules. There may very well be different materials in cementing substances than in non-cement tissues.

Mirsky: There has been a stupendous amount of work on the hematoparenchymal barrier as it applies to the central nervous system. Have there been any studies to indicate what particular changes occur with age, because we do know that during the early age of the organism certain substances and not others can pass through the barrier?

Angevine: Not that I know of. There is some investigation going on in this field, but I have seen no reports on it. The only area in which this connective tissue would occur in relation to the hematoparenchymal system in the brain is in the meninges, around the vessels in the Virchow-robin space and possibly in the choroid plexus, but there is no ground substance in the central nervous system as far as I am aware. In my opinion there may be a cement substance between the fibrils, but it has never been demonstrated to my knowledge.

Meyer: Gersh distinguishes a substance present in embryonal tissue which is soluble in water and which later on becomes insoluble It becomes soluble in certain pathological conditions.

Zweifach: Solubility alone would be a poor criterion for distinguishing between different constituents of the ground substance. Some of the intercellular cement substances are insoluble in a given pH range and become increasingly soluble at an acid pH. This may be interpreted as representing an increased ionization of these substances in the different pH ranges. Similarly, the possibility remains that these may be either sodium, calcium or potassium salts of the same protein complex. The calcium complex may be insoluble while the sodium or potassium salt will be soluble in water.

Meyer: The material that Gersh stains has different properties I do not know whether that in the histological sense means a barrier or a basement membrane, as Gersh calls it, but this area has

physical properties different in certain age groups or in certain times and in certain conditions.

Zweifach: From the physiological point of view, differences in the functioning of a given type of membrane or barrier are readily evident in comparing adult with embryonic structures. Thus, newly formed blood vessels in granulation tissue are more porous or permeable than are the same vessels several weeks or months later. As the vessel ages, it becomes less permeable to certain components of the blood, especially the blood proteins. The possibility exists that this process continues as the individual ages.

MacLeod: The vessel, or what is around it?

Zweifach: These changes would presumably occur chiefly in the pericapillary membrane.

Dempsey: May I belabor the adaptive business a little more? As Dr. Holbrook has mentioned, we are attempting to see whether there is any structural change in the connective tissue of the blood vessels which can be correlated with age. Age is only one physiological situation to which a tissue may be compelled to adapt. Normal development is another such situation, wound healing a third, and a hormonal stimulus still a fourth.

The first point concerns normal development. If phosphatase reactions are studied in the walls of blood vessels, little or no detectable activity occurs in embryonic tissues whereas in adults large amounts of enzyme are present in the walls of small blood vessels. The enzyme is located both in the endothelial linings and in the adventitial walls of the vessels.

In wound healing there is also a change in phosphatase. In normal, resting subcutaneous tissue there is little or no enzyme. During healing of a wound, phosphatase puts in an appearance and is present in high activity as scar fibers are forming, only to disappear again as the scar becomes organized.

Then finally, changes in phosphatase occur during different hormonal states. About a year ago we reported that the phosphatase located in the blood vessels of certain target organs disappears after hypophysectomy and reappears after replacement therapy (19).

Several years ago Burack, Wolfe and Wright (20) studied the collagen fibers of the uterus during the reproductive cycle. They showed that there is a great difference in the amount of fibrillogenesis occurring at different stages of the reproductive cycle. They demonstrated, by using ovariectomized animals injected with estrogen and progesterone, that the fibers demonstrable in the subepithelial portions of the uterus undergo a cyclic increase

and decrease even in an animal which does not menstruate. Thus, there are a number of situations in which a changed physiological state is associated with an altered morphology of the vessel wall.

Mirsky: Did you also not observe a diminution of phosphatase in tissues which were not under anterior pituitary regulation?

Dempsey: A diminution but not a change in pattern, in organs such as the salivary glands or kidneys. I was not sure how to interpret this observation. A hypophysectomized animal does not eat as much as does a normal one. If an animal is starved, there is a general decrease of phosphatase.

Jones. Dr. Dempsey's remarks bring me back to earlier discussions about the interrelationship of stucture and function, a question also pertinent to the problem of aging. It seems to me vitally important to keep in mind the fact that structure and function are measured by different methods. If the interpretation of the results of these different methods varies, then we must look for better methods which permit us to correlate functional with structural data. I presume that in the next few years the new hormone work will greatly increase our knowledge of the function of connective tissue. That does not necessarily mean we will have any new ideas about structure. Up to the present time our methods of visualizing structure by the use of various stains have both added and set a limit to our knowledge. It is extremely important to keep this in mind. Pathology today is almost completely dependent upon the evaluation of structural changes by methods now current in pathology. I am sure the pathologist would be the first to admit that this does not always work out. You may even find a connective tissue disease with fatal outcome where the pathologist by our present method would be hard pressed to find essential changes in structure. I have in mind disseminated lupus where at autopsy, as a rule, enormous connective tissue alterations are found, which make the continuation of function of the organs impossible. The next case, however, may have died with much the same clinical symptoms but with very little histologically detectable changes. I think that progress as related to structure has been blocked largely by long-established methods of inquiry, while in relation to function, there is just a small beginning due to better chemical understanding, better histochemical methods and others. We may hope that methods for the study of structure can be correlated with methods studying function. Then we could explain what changes take place in aging in connective tissue.

Holbrook: I would like to ask this question. If we are talking

about methodology, do we know all the salient facts about the structure of substance that can be learned by our newer techniques such as ultra-microscopy and x-ray diffraction? Is it the opinion of the group that that may lead to much more knowledge structurally or is it simply additional knowledge that is not going to add to our ultimate knowledge of function?

Angevine: In the first place, I agree with what Dr. Jones has said. That is why pathology departments are now concerned with function and chemistry, and are using the same methods that pathologists have been criticized for not using.

To answer Dr. Holbrook, one of the important things is first to reconcile some of the observations made with different techniques. If all investigators working on similar problems utilized identical techniques, their results would be readily comparable When two such investigators discuss a problem on which their results differ, it usually becomes apparent that they have used a different species of animal, different diets or different cultures so that their results may not be at all comparable. This feature is one of the greatest difficulties with all scientific investigation, but especially so in the realm of the biological sciences. It is also of the greatest importance to our evaluations here on this particular subject.

I also believe that investigation of disease can occasionally be restricted by the use of a particular technique One can start to use a specialized piece of apparatus and become a virtual slave to it. The investigator is frequently so busy keeping it in working order and developing it that he has no time for other investigations. More scientists are bound to a single technique or gadget than is generally realized.

We have constructed a new apparatus which I think may be of some value in this connection. It is a microradiographic apparatus, and we are just starting to use it One can measure the mass of any substance with an atomic weight of over 16 and can determine exactly in what part of the cell the chemical element is located. I believe that it may be applicable to a study of the interstitial tissues, and we may very well be able to localize many elements that are passing through the ground substance—especially those which have been well defined, such as calcium, phosphorus, also iron and iodine. I believe it may have a wide field of usefulness in this connection.

Meyer: May I bring up a problem which has been mentioned now a few times by Dr. Dempsey—the fibrillogenesis? I do not know whether this problem ought to be discussed here or with other problems.

Holbrook: Go right ahead.

Meyer: We proposed a theory of fibrillogenesis for which there is no experimental proof, namely, that the fibroblasts secrete large amounts of acid mucopolysaccharides together with a globular native protein, the precollagen. This native protein is precipitated and denatured onto the polysaccharide fibrils by local acid production by the cells, presumably by glycolysis, thus forming the fibrous proteins of the reticulum. The regularly spaced acidic group of the polysaccharide chain would form the template, so to speak, on which the fibrous proteins are built up. The mucopolysaccharides which first form a sheath on the fibrils would then be removed by enzymatic digestion.

Holbrook. Would you like to say a word about that?

Szent-Gyorgyi: I wanted to keep quiet because I disagree with most of what was said this morning, and I did not want to spoil the feast. I disagree even with the underlying idea of this Conference—it all depends upon what we call "medicine." On the one hand, medicine may be looked upon as an empirical subject dedicated to the relief of patients; an idea which I am willing to accept. However, on that basis we are just as far removed from science as the Peruvians were five centuries ago when they discovered quinine, and used it because it helped. On the other hand, medicine may also be looked upon as a science because in addition to interest in the end result of therapy we also want to understand *why* something is good for a patient. This is my way of looking at it. But if we want to understand, then we must go to the bottom of things. I am a bit frightened to hear structure deprecated at this Conference as something which does not tell us anything about function. There is no real difference between structure and function; they are the two sides of the same coin If structure does not tell us anything about function it means we have not looked at it correctly.

Structure can be studied in a wide range of dimensions. In the dimension of a centimeter, a heart valve is about 10^9 Angstroms. The human eye can see down to one-tenth of a millimeter, 10^6 Angstroms. The progress of medicine in the last century has been due in part to the discovery of the microscope, which takes us down to 10^3 Angstroms. But all of these structures which we see in these dimensions tell us little about function. The reason is that there are three more dimensions between this and the atomic level, between 1,000 Angstroms and 1 Angstrom, and it is in these dimensions that the macromolecules, the wheels of the living machine, lie. The secret of life is in this dimension. Chemistry

and anatomy are the same thing; chemistry is just molecular anatomy. Very important progress has been made in these last ten years through the introduction of the electron microscope which takes us down to about 30 Angstroms or so, into the macromolecular dimensions. We can actually *see* molecules now under the electron microscope. The structure which we see in the light microscope tells us very little. As we heard before, it does not even tell us the difference between an embryo and an old man of one hundred years.

Angevine: May I interrupt? I assume I am the one who has been referred to and believe that I have been misquoted. I have said that structure is important, but it is our opinion that the solution of the problem would be more adequately arrived at by devoting more of our attention to what lies between the fibers. Nobody has inferred that structure is of no importance, but we feel that the changes that take place in the structure in this instance are probably more or less secondary. I think a good illustration may be what occurs in coronary heart disease. If the blood supply to the myocardium is interfered with by a sudden occlusion of a vessel certain things happen to the muscle that cause death. However, such alterations are actually secondary to what has occurred in the blood vessel. The muscles are perfectly adequate, but the changes that take place in heart disease of this nature are secondary, nevertheless.

Fremont-Smith: Dr. Szent-Gyorgyi has overstated to make a point.

Angevine: Perhaps he did. I wanted to make this point before we got too far.

Fremont-Smith: Dr. Szent-Gyorgyi, we want you to go on.

Szent-Gyorgyi. One of the basic problems of morphology is the question of what is a fiber. You quoted Astbury as saying that a fiber is a long polypeptide chain. He is giving up that idea now. The electron muscroscopic work of Hall, Jakus and Schmitt (21) shows definitely that cross-striated muscle is composed of fibers; but those fibers are nothing but a row of almost round, slightly elongated particles held together probably by dipole moments in a long row. The striated muscle is in a way a crystal of particles about 300 Angstroms long and 100 Angstroms wide and the whole function must depend upon the field of forces surrounding these particles.

To my mind the next important question is how these particles are built and what decides the fields of forces around them. We have some information about this point since we know that

these particles are built of smaller globular units of 70,000 gm. molecular weight (Rozsa and Wyckoff). It seems that particles of this size are the only particles the system can elaborate. If it wants to build a fiber it makes one out of little round units of 70,000 gm. molecular weight. If the cell needs a larger particle of 300 Angstroms it puts them together from a dozen smaller particles. Probably the small round particle of 70,000 gm. molecular weight cannot develop the dipole moments necessary to withstand the strain to which the tissue is exposed. But if these units are put together in a definite way with atomic precision, then those dipole moments add up. If I put small magnets together in the right way the magnet moments will add up and I will get a strong magnet. So putting together those smaller globules may produce a larger particle with a strong dipole moment which will withstand the strain that muscle must withstand. In my opinion, tendon, connective tissue, and muscle tissue are all the same in this respect; the outer forces of the larger particles being decided by the way in which the smaller ones are put together. To make them join correctly certain substances such as ATP or perhaps cortisone must be present in small quantities.

For instance, just to give an example of the inaccuracy of the present microscopic method: Rozsa and Wyckoff have found in muscle around the fibers an exceedingly rich net of fibrils different from collagenous fibers but apparently related to them. This is of great importance in regard to the physics of muscle These fibers have a diameter of 200 Angstroms which is five times smaller than the limit of visibility under the microscope. There is a whole world in these dimensions which are beyond the limits of the common microscope. Structure and function are the same thing, only we must go to the correct dimension and not expect to find explanations where there are none. I wish the next Conference would put less emphasis on disease of connective tissue There has been at this Conference, some deprecation of normal structure and function and we have been told that it is necessary to start with the pathological I am entirely in disagreement with that. It is like the old joke of the man who when asked to repair a failing machine had to confess that he did not know how the machine worked. So if you don't know how it runs, how can you possibly know what is wrong with it?

I hope the next Conference will synthesize the two outlooks, the scientific and the empirical. Dr. Fremont-Smith emphasizes the synthetic idea. In my opinion this Conference started too much at the disease end. I would not make it entirely scientific

but I would start in the middle and develop the two views which would better represent the principles so very much in the forefront of scientific investigation — the dimensions below 1,000 Angstroms where the real morphological changes are revealed by electron microscopy, the ultraviolet microscope, and x-ray diffraction

Fremont-Smith What you have said is not in opposition to any preconceptions; it is just what we wanted to hear for it is only in this way I think that we can make progress. The one thing we don't want to do in these groups is to have, for example, only a fundamental group of people who are concerned at the electron microscope and x-ray diffraction levels or those who are working primarily on the chemistry of protein structure, and so forth What we do want to do is to bring together the people whose primary thinking has been clinical with those whose primary thinking has been at the other level to show that each must understand some of the dimensions of the other's thoughts This is necessary if we are going to have teamwork and joint progress

So I am delighted with what has been said because I think it [illegible] we need Please go on from there

[illegible] one remark However imperfect my words have been they were said in a friendly spirit. If I contradict it is to provoke discussion and to contribute to the subject I very often take the extreme view for that reason If we try only to smooth out contradictions there is no point in our getting together.

May I use a few more minutes to remark on the development of protein chemistry? Biochemists said: "We want to study proteins How can we get a protein? If we take a tissue, extract it with water, the protein will come out in great abundance and we will then study it." They did not notice that what they did was to separate two groups of protein which are different in principle, the soluble proteins, which have only secondary functions in the life forces, and the basic living structures, which are small globular particles such as the molecules of serum albumin which have to have a great mobility, to circulate and keep up certain physical constants in the blood. The great mass of living matter, to which life and individuality are linked, is in the solid phase; this structure, which was called "residue" was sent down the sink The molecules in these structures are interlaced in an intimate fashion, building large units, and cannot be separated without brutal methods which destroy the basic properties

these particles are built of smaller globular units of 70,000 gm. molecular weight (Rozsa and Wyckoff). It seems that particles of this size are the only particles the system can elaborate. If it wants to build a fiber it makes one out of little round units of 70,000 gm. molecular weight. If the cell needs a larger particle of 300 Angstroms it puts them together from a dozen smaller particles. Probably the small round particle of 70,000 gm. molecular weight cannot develop the dipole moments necessary to withstand the strain to which the tissue is exposed. But if these units are put together in a definite way with atomic precision, then those dipole moments add up. If I put small magnets together in the right way the magnet moments will add up and I will get a strong magnet. So putting together those smaller globules may produce a larger particle with a strong dipole moment which will withstand the strain that muscle must withstand. In my opinion, tendon, connective tissue, and muscle tissue are all the same in this respect; the outer forces of the larger particles being decided by *the way in which the smaller ones are put together.* To make them join correctly certain substances such as ATP or perhaps cortisone must be present in small quantities.

For instance, just to give an example of the inaccuracy of the present microscopic method: Rozsa and Wyckoff have found in muscle around the fibers an exceedingly rich net of fibrils different from collagenous fibers but apparently related to them. This is of great importance in regard to the physics of muscle. These fibers have a diameter of 200 Angstroms which is five times smaller than the limit of visibility under the microscope. There is a whole world in these dimensions which are beyond the limits of the common microscope. Structure and function are the same thing, only we must go to the correct dimension and not expect to find explanations where there are none. I wish the next Conference would put less emphasis on disease of connective tissue. There has been at this Conference, some deprecation of normal structure and function and we have been told that it is necessary to start with the pathological. I am entirely in disagreement with that. It is like the old joke of the man who when asked to repair a failing machine had to confess that he did not know how the machine worked. So if you don't know how it runs, how can you possibly know what is wrong with it?

I hope the next Conference will synthesize the two outlooks, the scientific and the empirical. Dr. Fremont-Smith emphasizes the synthetic idea. In my opinion this Conference started too much at the disease end. I would not make it entirely scientific

of function and structure, anatomy and chemistry as two difference principles.

I hope this Conference and this sort of conference will lead to an understanding of these principles.

Mote: There is some good in putting coins in a music box. Here you can see function alter rapidly such as the inflammatory reaction and connective tissue. As far as I know, no one has any idea what really happens to connective tissue or to other tissues when one of the hormones is suddenly given, but at least this can now be done and the next problem is to stimulate ideas at the fundamental level as to how far one may go along what systems of what disciplines to get at the basic mechanisms concerned in these tissue alterations.

Jones: I cannot understand why Dr. Szent-Gyorgyi feels that what he says is in any way different from the general tenor of what has been said here before. He has talked about the frontier where the new knowledge is going to come from. I said the same thing a few minutes ago when I talked about structure and function, and pointed out the limitations of the methods which have been in use for a long time and which are still being used. I did not mention the new and coming methods which have their limitations, too, nor do we know what we are going to learn from them I really believe that the combining of structure and function will naturally result as our methods improve in the various disciplines.

Szent-Gyorgyi: Yes.

Jones We cannot exactly see where the knowledge is going to come from and I don't think we have been talking here really from a clinical level.

Meyer: I am afraid that Dr. Szent-Gyorgyi's practical suggestions—the application of the electron microscope and of x-ray diffraction and the relation of structure to chemical composition —will not be too helpful at the present, either for the understanding of function of the connective tissue or for that of the brain cell. We have not reached the level yet even to speculate about such problems Let us take, for example, the action of cortisone on connective tissue. At present we do not know whether it acts on the cells, the ground substance or the fibrils of the connective tissue, or indirectly by its action on some other organ. Muscle is a comparatively simple system The muscle fiber contracts We can form hypotheses about this function by a few simple concepts of structure and change of such structure. In the connective tissue we are dealing with a system whose function we do not know.

I started muscle research many years ago, not because I think that muscle is especially interesting, but because I think it is one living matter of great importance and that different forms of life are but its slight adaptations: whether it is kidney or muscle is not interesting. The rapid motion in muscle makes it necessary that the structure have a simple geometry: it must be built of small particles linked together in one axis (the cross-axis) by forces only and arranged with great regularity. This provides the opportunity to disintegrate the whole system step by step into the smaller and smaller units. In this respect muscle is quite unique for this cannot be done with other tissues. It is possible to take the whole muscle down to fibers, the fibers to the fibrils, the fibrils to molecular filaments consisting of 100 to 300 Angstroms, the forces around which decide the behavior of the whole system. Then it is possible to go further and take these particles down to 70,000 gm. units which is probably the last unit. The next step would probably lead to amino acids Even more, we can reverse the process putting the smallest parts together again to form larger units; the larger units to fibrils; the fibrils to complex fibers which, in the end, jump as muscle does. So we can go down the scale, down to 30 Angstroms, the macromolecular level, and go back again and see where the real problems of life are buried These real problems, the explanation of the function of cortisone and hormones, are to be found here in these dimensions of 10 to 100 or 300 Angstroms. How these structures are put together will depend upon substances which must be present in minute traces and which supply the directing forces which fit them together.

Medicine is a queer science producing the most wonderful effect. For example, cortisone is amazing, but if you ask the doctor what cortisone really does, what the mechanism of its action really is, the doctor will have to confess his utter ignorance. The injection of hormones is similar to putting a coin into a music box; for a nickel I can get wonderful music and then say: "How clever am I?" The whole of medicine is in this stage. We are putting coins in the music boxes and out comes the music, but we have no idea of what we are doing. The reason is that we are in the wrong dimension. We missed the most important one which connects anatomy and chemistry. There is just one nature, as Dr. Fremont-Smith has pointed out. There is no function and structure; they are the same. There is no anatomy and chemistry; chemistry is anatomy at the atomic level. We will not understand all of these complex biological phenomena if we continue to speak

not out yet. The whole first chapter deals with this problem of organization, which is a most vital problem. What we mean by organization is that if we put two things together they get new qualities, and the whole is more than the sum of the two units. If I would start, for instance, making a watch from some iron salt, I would start by putting iron atoms together to a piece of metal. This piece of metal has entirely new qualities. These new qualities I can no more express in qualities of atoms at all, only in qualities of pieces of metal. Now from the piece of metal I make wheels and levers and what not. I get structures, which again can be described only in terms of wheels and no more in terms of metal. Then I put them together to make a watch which can be described now only in terms of a watch. Entirely new vistas and new properties come in at every new level. There are entirely new concepts; my watch already begins to show properties of living systems. The living have many more steps in organization.

So I agree with Dr. Mirsky, that science does not start nor stop with the atoms, nor does it start and stop with the human society. We may go still lower down to electrons, mesons, protons, and neutrons, or go higher up if we like. However, this "organization" is one of the basic ideas which has not been fully recognized in science, the philosophical aspect of organization, that any level and study of any level is equally justified. If I say we must extend our consideration to levels below 1,000 Angstroms, I don't say that that is everything. What is important is to know at which level we are, about which level we speak.

Mirsky: I am in accord with that.

Holbrook: That I think is an amazing discussion to arise out of the simple questions which I asked about the ultra microscope and x-ray diffraction, and whether we had exhausted our methods for studying structure. I presume, Dr. Szent-Gyorgyi, that you feel that the electron microscope and x-ray diffraction, and other methods to be developed may offer some hope of bringing together the two things where structure and function become the same?

Szent-Gyorgyi: I should like to add one more remark just to clear my position. Connective tissue is one level of organization. It is a very important unit, as such, which has to be dealt with as such. It is fine that we have symposia on connective tissue, although I don't like to have them just on the "diseases of connective tissue." If we take out of this tissue a fiber, this is again a new unit which is part of the larger whole, and which can be studied as an entirely new thing. You can measure its elastic

I am not arguing that we should not attempt to understand function from structure, but we may start at the wrong end.

Fremont-Smith: Perhaps, one thing that Dr. Szent-Gyorgyi is saying is that the basic function of muscle is the basic function of living cells. It is one example. It happens to be accentuated in a particular way, which gives it advantages for studying, but when we learn more about the function of the living muscle cell, we will already have some building blocks for the function of all living matter. Isn't that part of what you are saying?

Szent-Gyorgyi: Yes.

Fremont-Smith: I think Lawrence K. Frank, who has contributed so much to many things, said one time that function was structure changing through time. I think that is a pretty good phrase and perhaps helps to bring these things together.

Mirsky: I am disturbed. Two philosophic problems were raised by Dr. Szent-Gyorgyi. One refers to the concept of reduction, that the scientific method consists only in ultimate reduction. Where do we go from there? There is still going to be a reduction which man is not going to attain. He is always going to be able to conceptualize even about that which he cannot observe, and that brings us to another problem, which, according to Dr. Szent-Gyorgyi, would be unscientific for it is concerned with the individual who does the conceptualizing. I cannot conceive of expressing in dimensions of Angstrom units the mechanisms involved in the development of ideas which follow when people get together and talk about some subject. Understanding in terms of reduced "units" gives no clue to the mechanisms. I took issue and was unhappy with the statement that that is scientific which is seen in certain dimensions and not at other dimensions. It has been inferred that we have not gone down far enough in the range between 30 Angstrom units and 1, and that until we can get to that particular level of visibility we cannot be scientific. That is a question of methodology. The scientific approach need not deal with dimensions.

Fremont-Smith: I think the record will show an example of mishearing because I don't believe that Dr. Szent-Gyorgyi said—and I am going to ask him if he did—that there never was a question of scientific approach but rather the area or the dimensions in which certain very vital phenomena which we had to know about, and which would give us understanding of connective tissue function.

Szent-Gyorgyi: May I answer this question? I completely agree with Dr. Mirsky, and I am sorry that my book, just written, is

levels of organization. The question of normal and abnormal is a difficult one. Does the measurement of something at 100 Angstrom units or less provide a better measurement of what is normal or abnormal? These terms are introduced as values and I don't believe we need to introduce such concepts. When you administer cortisone or ACTH, you produce a change which is not necessarily a normal phenomenon. It is called normal only because of the value aspect, i.e., because the patient gets better and can now walk, has a more mobile joint, and so forth. He is now "normal" with respect to certain standards.

Fremont-Smith: "With respect to," I think that solves the problem.

Mirsky: At any particular dimension.

Holbrook: I think Dr. Mote's point is well taken, that it is of some importance both in stimulating curiosity and demanding work to determine the mechanism by which the watch is repaired, and you cannot help but get excited about the mechanism by which the thing happens.

Jones: There is one other aspect. It seems to me we have to work both ways. We cannot start today from an atom or a group of atoms and make a man, so we have to use man, and literally use him and his component parts in order to learn more about it. I dor[illegible]
to [illegible]
kn[illegible]
than from another.

Meyer: Perhaps we can bring the discussion back from where it started. I asked the question whether there is any information about the mechanism of the transformation of a soluble protein, the precollagen, into the insoluble connective tissue fibers.

Szent-Gyorgyi: That question is simple to answer. We have no information as yet. I want to add a last word. I sense a note of misgiving in Dr. Mote's words; he feels I depreciated disease. To my mind disease is the most wonderful experiment that nature makes which we can not make ourselves, and it gives us new clues. I agree with Dr. Fremont-Smith that we have to use all approaches. Cortisone and ACTH are wonderful, and Dr. Mote played an important part in their development. I have all the appreciation and admiration for that work, and the human side of this discovery. However, we cannot stop at the connective tissue level; if we want to understand we must go to lower, and also to higher levels. As to my watch, which Dr. Angevine does not

properties, put it under the electron microscope and x-ray and see the periodicity; you can pull it down to let us say 70,000 gm. units. That, again, is a new unit. For 70,000 gm. one must apply entirely new methods, as is necessary at any level, but in the end, when I have done all that, and have gone down to atoms, then only will I have an idea what the connective tissue is, whereas if I just study "connective tissue," as such, I can never understand it. That is just one level. We have to have all levels and not exclude that dimension below 1,000 as we have done here.

Fremont-Smith: All levels, with the integration at all levels.

Szent-Gyorgyi: Evidently.

Fremont-Smith: All levels must be pulled together.

Szent-Gyorgyi: If we don't go down to atoms, and come back, we will not understand the higher level.

Angevine: In that discussion about the building of the watch you had to know how to construct that watch, which was no mean feat. You had a model of a watch that you started to build.

Fremont-Smith: We have a model of a man or a model of a frog.

Angevine. You must have something visualized before you start down; in other words, there must be direction.

Fremont-Smith: And it is usually when the watch goes wrong or when the nickel dropped in music machines brings out jangles, and machines won't run, that people begin to study with great intensity. That is the point, that we may go back to the beginning again, not that the abnormal is more important to study than the normal, but the approaches to the normal are frequently made through observations from the deviations from the normal, and the student can learn more of the normal from the study of deviations than he can by a frank approach to the normal, which is the most difficult of all studies.

Szent-Gyorgyi: Yes.

Mote. I think one of the most exciting things about this present era is that the connective tissue watch breaks down all of a sudden. You can in a sense and to a degree repair it with a hormone now. That certainly gives rise to a double approach, not only the normal and the abnormal, but shifting back and forth between the two more or less at will, I think that is a great advantage in this particular period. It will allow one to have more imagination on what attacks to take, than if you had to study either one or the other alone.

Mirsky: I see we are now really in accord as to dimensions and

14 EPPINGER, H *Permeabilitats Pathologie* Wien Springer-Verlag, 1949

15 HARMAN, J W Studies on mitochondria I The association of cyclophorase with mitochondria *Exp Cell Research* **1**, *382 (1950)*

16 HARMAN, J W, and WEBSTER, J H Effect of aging on the protein fractions of the normal human heart *Federation Proc* **8**, *357* (Abstract) *(1949)*

17 VOLTERRA, M Einige neue Befunde uber die Struktur der Kapillaren und ihre Beziehungen zur "sogenannten" Kontraktilitat derselben *Zentralbl f inn Med* **46**, *876 (1925)*, Ricerche sul sistema redicolo-istiocitario *Lo Sperimentale* **81**, *319 (1927)*, Ulteriori studi sulla morfolgia e sulla funzione dei capillari sanguigni *Arch ital di anat e di embriol* **33**, *844 (1934)*

18 GOREV, N N, and SMIRNOVA-ZAMKOVA, A J Observations on pathogenesis of hypertension *Am Rev Soviet Med* **3**, *28 (1945)*

19 DEMPSEY, E W, GREEP, R O, and DEANE, H W Changes in the distribution and concentration of alkaline phosphatases in tissues of the rat after hypophysectomy or gonadectomy, and after replacement therapy *Endocrinology* **44**, *88 (1949)*

20 BURACK, E, WOLFE, J M, and WRIGHT, A W Effects of administration of estrogen on connective tissues of genital tract of rat *Endocrinology* **30**, *335 (1942)*

21 HALL, C E, JAKUS, M A, and SCHMITT, F O Investigation of cross striations and myosin filaments in muscle *Biol Bull* **90**, *32 (1946)*

quite like, I will readily admit that there is one difference in methodology; we can build a watch, but we cannot make a man (not synthetically, the hard way, at least). The only way left is to break him down, but I am glad to say we can build up a little way, too.

Angevine: I like the watch, but I think we should know what we are building.

REFERENCES

1 CLARK, F. L *The Tissues of the Body* Oxford· Oxford University Press, 2nd ed., 1945.

2 JORDAN, H E. *A Textbook of Histology.* New York and London Appleton-Century, 8th ed, 1946-1947.

3 MAXIMOW, A W, and BLOOM, W.· *A Textbook of Histology* Philadelphia and London W. B Saunders Co, 4th ed, 1942

4 GROSS, J A study of the aging of collagenous connective tissue of rat skin with the electron microscope. *Am J. Path.* **26**, *708* (Abstract) *(1950)*

5. STEARNS, M L Studies on development of connective tissue in transparent chambers in rabbit's ear. *Am. J. Anat* **67**, *55 (1940)*

6 GERSH, I, and CATCHPOLE, H. R: The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth *Am. J. Anat* **85**, *457 (1949)*.

7 FISCHER, A *Biology of Tissue Cells.* Cambridge, England· Cambridge University Press New York G E Stechert and Co (Translated from the Danish by Einar Christensen), 1946.

8 PLOTZ, C M, HOWES, E. L, MEYER, K, BLUNT, J. W., LATTES, R., and RAGAN, C· The effect of the hyperadrenal state on connective tissue. *Am J Path* **26**, *709* (Abstract) *(1950)*.

9 SPAIN, D M, MOLOMUT, N, and HABER, A: The effect of cortisone on the formation of granulation tissue in mice *Am. J. Path* **26**, *710* (Abstract) *1950*

10 WOLBACH, S B., and BESSEY, O A: Tissue changes in vitamin deficiencies *Physiol Rev* **22**, *233 (1942)*.

11 ELSTER, S K Effect of ascorbic acid deficiency on the collagen content of guinea-pig tissues. *Am. J. Path* **26**, *711* (Abstract) *1950*

12 ALTSHULER, C H, and ANGEVINE, D. M· Acid mucopolysaccharide in degenerative tissue of connective tissue, with special reference to serous inflammation *Am J. Path.* (to be published).

13 RÖSSLE, R Uber die serosen Entzundungen der Organe *Virchow's Arch. f path. Anat.* **311**, *252 (1944)*

examples of this change.

The third question: Does the occurrence of fibrinoid change imply that the diseases in which it occurs are related etiologically or pathogenetically? The mere listing of the disorders in which it is seen would seem to answer this question in the negative, at least as far as etiology is concerned. Certainly the change appears in diseases that are unrelated. With respect to the pathogenesis of fibrinoid, we need to exercise caution, for until much more has been learned, we have only a vague idea as to what the change is or how it is developed.

Does the alteration in tissue imply a specific change in the chemical sense? This was my fourth question. My purpose in raising this question is to initiate discussion from members of this Conferei

Now

methods

only cru

at the present time. It would be highly important if we knew how to define this change, about which there is so much interest, in terms of chemical alteration. Let me say again that we do not know the answer; at least, I don't. It would seem that, in considering pathogenesis of fibrinoid, there are a number of factors that should be taken into account, and the thoughts that I am going to express may have a bearing on this morning's discussion as to what constitutes connective tissue.

In considering the pathogenesis of fibrinoid change, we have to consider the special properties of the tissues in the sites in which fibrinoid occurs. One example of what I have in mind is the degree of vascularity of a part Connective tissues in some areas are highly vascular. Here the vascular-parenchymal barrier is brought into play much more frequently than in other structures where the connective tissues are dense and far removed from vascular structures There are many relationships of this sort that enter into the capacity of a tissue to react. In the pathogenesis of fibrinoid change, such factors are doubtless of great importance and must be taken into account. In highly vascular structures, such as the lungs, there is an opportunity for a more rapid exchange of substances between blood and tissue fluids than exists, let us say, in the skin. Does the extravasation of plasma into tissues influence fibrinoid change? There have been some observers who have assumed that fibrinoid change is nothing other than the imbibition of plasma protein in the connective tissue fibrillae. Others have concluded that it represents an exudation of fibrin in the

I think that the credit for the term goes to E. Neumann (2) who described what is now called fibrinoid degeneration or fibrinoid change in a variety of inflammatory processes, including serous, synovial, and mucous, that occurred principally on surfaces. Apparently what he had in mind embodied almost all we know today —there are a few exceptions which we will point to later—it was something that was acidophilic or eosinophilic in its staining properties, it was acellular, it was homogeneous and refractile, and it had a fibrin-like quality to it as one viewed it through the microscope. Probably through the influence of F. Klinge the change took on importance, because in Klinge's monograph on rheumatic disease (3) he stressed this change as being the essential lesion in rheumatism. Following his description of the fibrinoid change, there have been numerous papers which have referred to it in relation to other disease syndromes and entities, including rheumatoid arthritis, disseminated lupus, periarteritis nodosa, and a wide variety of other conditions. From more recent observations we can add to what Neumann had in mind by saying that this material has a property of metachromasia, as Dr. Angevine disclosed this morning. It also has a property of staining intensely by methods employed for the demonstration of mucopolysaccharides.

My second question was: In what clinical and pathologic states does the change occur? I have already named a few. We see this change occurring conspicuously and consistently in rheumatic fever. We see it well displayed in most examples of disseminated lupus erythematosus. We see it in certain examples of rheumatoid arthritis, in periarteritis nodosa, in scleroderma and dermatomyositis, and we see it in the vascular lesions of thromboangiitis obliterans. These are the principal disorders in which the change occurs in some parts of the body. However, we see what morphologically appears to be a similar alteration in a number of isolated lesions that seemingly are not associated with a generalized disorder of connective tissue. For example, we see it very well demonstrated in isolated bursae that have been traumatized or injured. We see it—and this was known very soon after the process was first described—in the bases of peptic ulcers. We see something that looks very much like it in placental tissue. It has been alleged to be the same type of change that is seen in glomerular and arteriolar structures, in arteriolonecrosis or arteriolosclerosis. Then we see it in the lung in a number of conditions in which there seems to have been prolonged extravasation of protein-rich fluids. Here we see the material plastered against the alveolar walls, and it undergoes organization. Those are the common sites in which we see

examples of this change.

The third question: Does the occurrence of fibrinoid change imply that the diseases in which it occurs are related etiologically or pathogenetically? The mere listing of the disorders in which it is seen would seem to answer this question in the negative, at least as far as etiology is concerned. Certainly the change appears in diseases that are unrelated. With respect to the pathogenesis of fibrinoid, we need to exercise caution, for until much more has been learned, we have only a vague idea as to what the change is or how it is developed.

Does the alteration in tissue imply a specific change in the chemical sense? This was my fourth question My purpose in raising this question is to initiate discussion from members of this Conference who may be qualified to guide our thinking

Now let us consider the pathogenesis of fibrinoid The staining methods that have been available to morphologists thus far are only crude tools, and I do not believe we can answer the question at the present time It would be highly important if we knew how to define this change, about which there is so much interest, in terms of chemical alteration. Let me say again that we do not know the answer, at least, I don't. It would seem that, in considering pathogenesis of fibrinoid, there are a number of factors that should be taken into account, and the thoughts that I am going to express may have a bearing on this morning's discussion as to what constitutes connective tissue.

In considering the pathogenesis of fibrinoid change, we have to consider the special properties of the tissues in the sites in which fibrinoid occurs One example of what I have in mind is the degree of vascularity of a part Connective tissues in some areas are highly vascular. Here the vascular-parenchymal barrier is brought into play much more frequently than in other structures where the connective tissues are dense and far removed from vascular structures. There are many relationships of this sort that enter into the capacity of a tissue to react. In the pathogenesis of fibrinoid change, such factors are doubtless of great importance and must be taken into account. In highly vascular structures, such as the lungs, there is an opportunity for a more rapid exchange of substances between blood and tissue fluids than exists, let us say, in the skin Does the extravasation of plasma into tissues influence fibrinoid change? There have been some observers who have assumed that fibrinoid change is nothing other than the imbibition of plasma protein in the connective tissue fibrillae. Others have concluded that it represents an exudation of fibrin in the

connective tissue with precipitation around the collagen fibrils, adding to them qualities of acidophilia and refractility. The view has also been expressed that fibrinoid actually represents a retrograde change, a process of degradation of connective tissue itself, of collagen and ground substance. Then there has been the idea—perhaps this is the one that Dr. Angevine adheres to—that fibrinoid change represents the precipitation of acid mucopolysaccharides in the ground substance of connective tissue, changing the structure and perhaps the molecular composition of the ground substance of mesenchymal tissues.

In thinking about the pathogenesis of fibrinoid change, we should try to define more clearly what we mean by the term. I am going to show some lantern slides a little later on in which a great variety of changes or, at least a great number of stages of change, have occurred. All of these are commonly spoken of as fibrinoid change, and it is a little difficult to believe—if we accept all of the changes under this one term—that we are ever going to define the composition of fibrinoid or explain its pathogenesis. Perhaps we should comment on the stages of development of the process.

What are the morphologic stages in the development and regression of fibrinoid change? This was my sixth question Again, this is a very difficult question to answer for the same reasons that have been enumerated. It would seem to me that the first alteration is represented by an increase in the volume of the material that lies between the fibrillar structures of connective tissue. With that increase, there occurs a dispersion of the connective tissue fibrillae. At this time there appears to be an increase in metachromasia of the tissue and this can be demonstrated with toluidine blue. The fibrils themselves appear abnormal. To me, they appear swollen.

Angevine. May I interrupt?

Bennett: Yes.

Angevine. In most collagen fibril preparations I think that is usually accepted, but last week in Madison, Gale (4) described collagen fibrils with the electron microscope from a great variety of diseases—most of the diseases you have enunciated. He came to the conclusion there was no essential difference in the structure of the fibril from diseased and normal tissues.

Bennett: Would you agree that when you look at a lesion with the light microscope the altered fibrils appear granular?

Angevine: I think I would. I was citing his observation, which

I was not previously aware of.

Meyer: Maybe we should remember here that hydration as seen under the light microscope might not show up under the electron microscope. The collagen of the rat tail tendon brought into solution by dilute acid and then reprecipitated by salt or neutralization shows no difference from native collagen fibers under the electron microscope, according to F O. Schmitt (5).

Bennett: We might mention that the electron microscope is demonstrating much smaller units. What you see under the light microscope is an arrangement of these small units but not the individual units.

At least that is the sequence of fibrinoid development as we see it in tissues that have been harshly treated by fixatives and then sectioned and stained. It acquires that appearance—at least I think it does—of being swollen, hyaline and granular, and often fragmented. When we apply an appropriate stain we see mixed with the fibrillar structures that were there before, a fibrillar substance that stains in a manner identical with that of fibrin itself Phosphotungstic acid-hematoxylin stain, let us say, on such lesions will oftentimes show a blue-staining fibrillar material that is interdigitated with or superimposed upon the material that we believe, because of staining properties, to be the original connective tissue substance. Then, from this stage on, the lesions become rather more grossly changed. There is imparted to them a homogenicity and refractility and very strong acidophilic staining property, apparently this can continue on to complete disintegration In this final stage you have only amorphous granular debris left If we include some of the larger lesions which are shown in Figures 1-17, this process may not even stop there but may go on ultimately to liquefaction of the material.

There are other things that happen in conjunction with this process which may or may not be a part of it. The fibroblasts which happen to be located in an area in which this change occurs take on abnormal forms and configurations. They become swollen Their nuclei become enlarged. The nucleolus becomes markedly swollen and centrally placed, giving the nuclei a sort of bird's-eye appearance, and the tissues around the margins of the lesions undergo varying degrees of change. These vary from slight to very marked edema. There is an infiltration with migrating cells, both polymorphonuclear leukocytes and macrophages. In time, if our concept of sequences is correct, the cells at the periphery of these lesions become oriented in a regular direction, pointing toward the central necrotic focus. This we designate as palisading.

connective tissue with precipitation around the collagen fibrils, adding to them qualities of acidophilia and refractility. The view has also been expressed that fibrinoid actually represents a retrograde change, a process of degradation of connective tissue itself, of collagen and ground substance. Then there has been the idea—perhaps this is the one that Dr. Angevine adheres to—that fibrinoid change represents the precipitation of acid mucopolysaccharides in the ground substance of connective tissue, changing the structure and perhaps the molecular composition of the ground substance of mesenchymal tissues.

In thinking about the pathogenesis of fibrinoid change, we should try to define more clearly what we mean by the term. I am going to show some lantern slides a little later on in which a great variety of changes or, at least a great number of stages of change, have occurred. All of these are commonly spoken of as fibrinoid change, and it is a little difficult to believe—if we accept all of the changes under this one term—that we are ever going to define the composition of fibrinoid or explain its pathogenesis. Perhaps we should comment on the stages of development of the process.

What are the morphologic stages in the development and regression of fibrinoid change? This was my sixth question. Again, this is a very difficult question to answer for the same reasons that have been enumerated. It would seem to me that the first alteration is represented by an increase in the volume of the material that lies between the fibrillar structures of connective tissue. With that increase, there occurs a dispersion of the connective tissue fibrillae. At this time there appears to be an increase in metachromasia of the tissue and this can be demonstrated with toluidine blue. The fibrils themselves appear abnormal. To me, they appear swollen. They appear refractile. Very often they are granular, and in many instances they appear fragmented or partially disintegrated. If we apply a phosphotungstic acid hematoxylin stain, —

Angevine: May I interrupt?

Bennett: Yes.

Angevine: In most collagen fibril preparations I think that is usually accepted, but last week in Madison, Gale (4) described collagen fibrils with the electron microscope from a great variety of diseases—most of the diseases you have enunciated. He came to the conclusion there was no essential difference in the structure of the fibril from diseased and normal tissues.

Bennett: Would you agree that when you look at a lesion with the light microscope the altered fibrils appear granular?

Angevine: I think I would. I was citing his observation, which

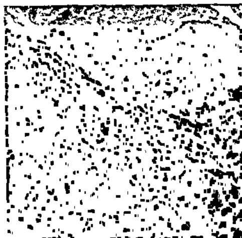


FIGURE 1



FIGURE 2

The lesion shown in Figure 1 is the well-known myocardial Aschoff nodule. It occurs, as most of you know, in connective tissue septa within the myocardium. The Aschoff nodule consists of more than fibrinoid change. There are two essential components to it. One is change in connective tissue, the so-called fibrinoid change, and the other is the cellular response. We see in the center of the lesion the fibrinoid change which has led to considerable spacing, and around that we see an arrangement of swollen hyperactive cells. Sometimes we see these cells undergoing mitotic division.

The Aschoff nodule varies markedly with its stage of development and perhaps with its age. Also it may vary according to the degree of activity of the di
2 it is easier to see what has
vessel on the left-hand side
the connective tissue fibrils have been dispersed. They have become swollen, and they have become overlaid with material that does not show too well in this photograph, but under the microscope you would note that it is acidophilic and granular. In between these fibrillar structures we see very active-appearing mesenchymal cells. These have large swollen nuclei and prominent nucleoli. I wish to emphasize this point because the Aschoff nodule is made up of both a fibrinoid change and a cellular response. I would like to point out here that we are dealing with lesions that tend to occur close to the walls of blood vessels and that they tend to occur in rather vascular structures.

The rheumatic subject presents other cardiac lesions. We see not only the nodules within the myocardium that we have just illustrated but, in addition, we see the changes in the pericardium. If you look closely at these sections, you will see that on the surface there is fibrin. (See Figure 3.) This fibrinous exudate is engrafted upon connective tissue in which fibrinoid change has occurred.

The reproduction shown in Figure 4 is from the posterior wall of the left auricle, where the histologic changes are apt to be very striking. We see marked thickening of the entire endocardial and subendocardial layers. The connective tissues have acquired an acidophilic staining quality. There is a certain amount of latticing of the connective tissue substance and a certain amount of layering of cells between the latticed connective tissue. If this were stained in the manner that Dr. Angevine referred to this morning, we would find an increase in metachromasia in the ground substance.



FIGURE 3

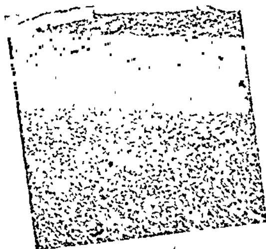


FIGURE 4

In other parts of the body—we leave the heart for a minute—in the rheumatic fever subject, we see changes in connective tissues. These changes involve both the intercellular and cellular elements. Along with numerous observers, we (6) have described these changes in detail.

Next I would like to illustrate a few of the lesions that are seen in rheumatoid arthritis

Figure 5 shows a section of a subcutaneous nodule showing multiple foci of fibrinoid necrosis. It is because of the pronounced differences between these lesions and those we have just demonstrated in rheumatic fever that I have referred to this change as fibrinoid degeneration or fibrinoid necrosis.

Figure 6 shows an example of an early nodule. In the upper part of the field is fibrinoid change. In the lower half of the slide we see connective tissue which has become more cellular. Also we see interstitial substance which is more distinct than formerly. The collagen fibrils are dispersed and swollen. In the middle zone, running slightly diagonally across the field we see what is commonly called fibrinoid change.

If we apply a stain to demonstrate glycoproteins we see in Figure 7 an intensification of the stain at the margins of the necrotic zones. This alteration in staining property may be due to a pronounced change in the ground substance.

Angevine: Is the highly refractile body elastic tissue?

Bennett: Yes, I think those are elastic fibrils.

The reproduction in Figure 8 shows a fully developed rheumatoid nodule. The entire central area has become necrotic and disorganized. Cells have disappeared from the necrotic part of the lesion. The marginal cells are now oriented in a palisade fashion. The debris in the center of these lesions undergoes further regressive changes and eventually may liquefy.

Now I would like to illustrate a few examples of fibrinoid change as it is seen in disseminated lupus.

Figure 9 is a lesion in the skeletal muscle tissues of a patient with lupus. Adjacent to the small blood vessel coursing diagonally across the field is a lesion that looks somewhat like an Aschoff nodule. This is a form of fibrinoid change.

In the joints of patients with disseminated lupus we frequently see changes in the synovial and subsynovial tissues. In Figure 10 there is shown hyperemia and mild cellular infiltration. The connective tissue beneath the synovial lining shows coarsening of the fibrillar structures, and there is increased spacing between



Figure 5

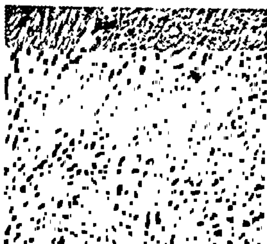


Figure 6

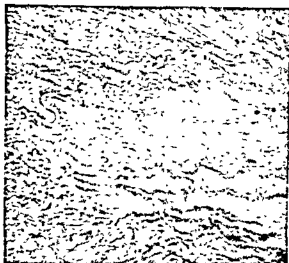


FIGURE 7



FIGURE 8



FIGURE 9

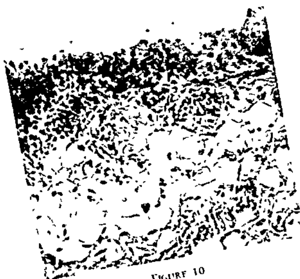


FIGURE 10

collagen bundles which may be a result of changes in the ground substance.

I have been impressed by the fact that in several cases of disseminated lupus there have been rather striking synovial membrane lesions of "fibrinoid" character. The next four slides were made from the tissues of an affected joint.

In this hematoxylin-eosin stained section (Figure 11), there is engrafted upon the synovial surface an acidophilic material that looks like fibrin. It actually extends down into the tissues and is noted between the synovial cells. This material is in part hyalin in quality, but it is partly granular.

When a phosphotungstic acid-hematoxylin stain is applied we note that the material is not uniform in staining properties. (See Figure 12.) Some parts stain orange or yellowish while other fibrillar parts stain intensely blue.

If we apply another stain we get different colors, but again the material seems to intermingle with the synovial cells. Then we notice that beneath the synovial intima the ground substance has seemingly increased in amount, or perhaps has become more fluid in character—at least there is increased spacing between the connective tissue fibrils.

With the aniline blue stain, we find that the material on the surface is stained an intense red. This material surrounds and/or is interlaid between the blue-staining collagen fibrils. (See Figure 13.)

The section shown in Figure 14 has been stained for glycoprotein. In the zone showing fibrin deposit and fibrinoid change, there is a pronounced red coloring.

Lastly, I would like to present three figures illustrating the common lesions of periarteritis nodosa.

Figure 15 shows a segment of a coronary artery. The vessel wall shows inflammatory and degenerative changes. The media, adventitia and surrounding connective tissues all show the type of change that is usually designated fibrinoid degeneration.

Also in the smaller vessels within the myocardium we may observe fibrinoid changes close to blood vessel walls. These lesions are frequently noted at the bifurcation of vessels. The fibrinoid change is accompanied by a cellular infiltration and edema of the adjacent connective tissue as shown in Figure 16.

Similar vascular lesions may result in thrombosis, as is seen in Figure 17.



FIGURE 11



FIGURE 12

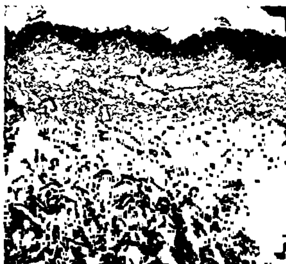


FIGURE 13



FIGURE 14

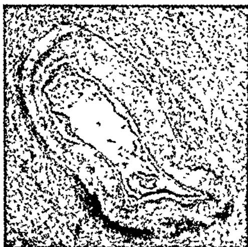


FIGURE 15



FIGURE 16

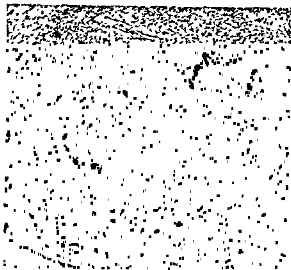


FIGURE 17

Fremont-Smith: You get a very similar picture to that in thromboangiitis.

Bennett: A similar picture with respect to fibrinoid. In that disease the change is predominantly in the interior of the vessel

Fremont-Smith: Fibrinoid?

Bennett: Yes. Fibrinoid.

Finally, I wish to show a copy of the colored illustration in Altshuler and Angevine's paper* — I think Dr. Angevine has already shown the two illustrations at the top to demonstrate the disappearance of the metachromasia in the hyaluronidase-treated section. I refer to it only to direct attention to it in the event Dr. Angevine wishes to comment on it in discussion.

Other examples of fibrinoid change that I might have illustrated include examples of scleroderma, dermatomyositis, and a few other lesions. The basic changes have been pretty well illustrated in the reproductions of similar lesions in other diseases mentioned and I will stop here.

Holbrook: Dr. Dempsey, do you have something you want to say?

Dempsey: I have a few things that I thought might be of interest. They are, in a sense, additional remarks to some that Dr. Bennett made. He mentioned the sites at which fibrinoid occurs,

*See page 1077 in *Am J. Path* 25 (1949)

and I noted that he included in them the placenta Our laboratory, particularly Dr. Wislocki, has been interested in this region for some time, and has made observations upon the fibrinoid there (7).

It is an interesting situation for two reasons One of them is that, surrounding and permeating the cytotrophoblastic cell islands and cell columns, there is frequently an area which stains differentially with procedures such as the Mallory tri-acid and Azan methods. The peripheral part stains red, like fibrin, but the central part takes a bluish coloration. This portion is the so-called fibrinoid. It is acidophilic, as Dr. Bennett mentioned, and it also reacts intensely when subjected to the periodic acid-Schiff procedure. It is an interesting region because there is no collagen in the cytotrophoblastic cell islands and columns. Hence, here we can abstract at least one element from this complex called fibrinoid. Since it gives the characteristic reactions of fibrinoid as studied in other locations, we can at least be sure that collagen is not a necessary component for the production of the characteristic appearance.

We have studied still other staining reactions of this fibrinoid. In recent years, we have devised a very carefully controlled method of staining with acid and basic dyes. The variables which influence dye-uptake are the concentration of dye, the length of time staining is carried out, the ionic strength of the staining bath, the pH of the staining bath, and the type of fixation employed. If one controls all five of these variables, one obtains reproducible staining. We have controlled four of them and have deliberately varied the pH of the bath. Basic dyes stain intensely in alkaline solutions, but as the pH is lowered, there comes a point, different for different tissue components, at which staining no longer occurs. Conversely, with acid dyes, intense staining occurs in acid reactions and fails as the pH rises. Thus, we can describe basophilia and acidophilia as a function of pH When these procedures are applied to the placenta, we find that both the fibrin and the fibrinoid stain identically; that is, the pH at which abolition of staining occurs is exactly the same for both.

Furthermore, I mentioned that fibrinoid gave a positive reaction after treatment with periodic acid and the Schiff reagent. We observed that fibrin, either in this placental location, or in clotted blood, gave an identical reaction. However, since this reacti might have been due to the presence of some adventitious co ponent of the blood clot (say a glycoprotein), we procured . purified fibrinogen from Dr. Cohn's laboratory, clotted thrombin and made a film of it. The film, when tested

periodic acid-Schiff reagents, stained as brilliantly as did the fibrin or fibrinoid in the tissue section.

Meyer: How much carbohydrate was contained in the fibrin film as judged by the carbazole reaction of Dische or by analysis for hexosamine?

Perlmann: I would like to point out that periodic acid is not specific for carbohydrates. Dr. Goebel and I have studied the reaction of periodic acid with proteins and found that several amino acids of the protein are oxidized by periodate.

Dempsey: I don't know the answer to your question, Dr. Meyer, because we did not determine the carbohydrate content. However, this was purified fibrinogen, which I believe they state is 99 percent fibrinogen.

I don't want to debate the que:
is due to the one-two glycol linl
morning, but merely to indicate
these additional reactions, there was no difference between fibrinoid and fibrin in the placenta.

Now, there is still another line of evidence that can be brought to bear on this question. This concerns the physical consistency of the material, and the mechanism of the tri-acid stains. The consistency, or the coarseness of the feltwork of the fibrin clot, can be made to vary depending upon the conditions under which it is formed If the clot forms at one pH, the fibrils are coarse, and at another pH they are fine. Thus, one can prepare artificially fibrin films which have the same microscopic differences in structure which characterize fibrin and fibrinoid as they occur in sections.

Now, with respect to the Mallory connective tissue stain. It is a procedure which is so complex, chemically, as to defeat entirely any attempts of mine to understand it. The red and blue colorations are due to the presence of aniline blue, in solution, and azocarmine, in suspension. In the staining process, one changes the pH rather arbitrarily and very drastically at one stage of the procedure. Then finally, it is not a procedure which goes to completion, but rather one which the histologist controls by differentiation—that is, he regulates the intensity of the colors until they satisfy his preconceived notion of how they ought to look. All of this indicates that we should place no reliance on chemical differences as evidenced by altered staining with such procedures. Rather, the suspicion is raised that these dyes stain perhaps by entrapment of the suspended dye-particles in a net of particular size. Indeed, I believe Hardy first suggested that the di- and tri-

acid staining procedures indicated only a changed physical state of the protein. Thus, there is an alternative explanation for the staining reactions which does not imply a chemical difference between fibrin and fibrinoid.

Holbrook: That is very important.

Meyer: May I ask, you believe there is no difference between fibrin and fibrinoid?

Dempsey: Personally, I think there probably is not, but I concluded only that we could not demonstrate any difference by these techniques.

Bennett: Does that not point to a need for specifying what we have in mind when we say fibrinoid? Are we talking about the material in the placenta which you refer to and which appears very similar to the substance I demonstrated on and in the lining membrane of the articular cavity in the patient with disseminated lupus, or are we talking about the material observed in the Aschoff nodule, or are we speaking of the material seen in the necrotic centers of the subcutaneous nodules in rheumatoid arthritis? The all-inclusive uses, to which these terms "fibrinoid change," "fibrinoid degeneration," or "fibrinoid necrosis" have been put, almost preclude the possibility of precise interpretation unless we define the term more precisely and build up our conception of the process little by little. Furthermore, I think we must consider the term in association with the type of tissue in which the change occurs, for highly vascular tissue like synovia may respond to an injury very differently from dense and non-vascular structures like the heart valves, yet we designate the resultant alteration in both as fibrinoid change.

Mote: The Aschoff body always struck me as looking like fibrin as far as fibrinoid change is concerned.

Meyer: Another question as far as technique is concerned, Dr. Angevine, did you also use the Hotchkiss stain?

Angevine: Yes, we did

Meyer: The fibrin stains remained after treatment with testicular hyaluronidase?

Dempsey: I think the present status of testicular hyaluronidase is a little unsatisfactory.

Meyer: Hyaluronidase is not a pure enzyme but you could make fairly sure the absence of some contaminating enzymes.

Fremont-Smith: Would you restate your definition so that we have it clear?

Meyer: You can prepare hyaluronidase free of any demonstrable proteolytic effect or you could test the commercially available

samples for the absence of proteolytic action.

Fremont-Smith: Then if it does, what?

Meyer: It would be of interest to find whether such a preparation still removed something which shows metachromatically and takes the Hotchkiss stain.

Fremont-Smith: You say that is fibrinoid or not?

Meyer. I would like to stay clear from any definition because I do not know enough about the nature of the fibrinoid change. I would like to find out, however, whether there is a physical or a chemical difference between fibrin and fibrinoid.

Dempsey: The point is, Dr. Angevine showed that the metachromasia of the region was destroyed by hyaluronidase and I said that the periodic acid-Schiff reaction of fibrinoid also occurred in what is known to be fibrin. Then the question is, whether the periodic acid-Schiff reaction is also destroyed by hyaluronidase or not. It may be the mucopolysaccharide that is giving the metachromatic effect. Perhaps Dr. Angevine has done the experiment. I haven't, and I don't know.

Meyer. It may or may not digest it.

MacLeod: When you digest away the polysaccharide with hyaluronidase how much protein is liberated? When the enzyme removes the staining property can one be sure that it is the polysaccharide that is giving the reaction? May not the protein wash out when the polysaccharide is destroyed?

Meyer: This is quite possible and, in fact, probable since the solubility of the protein-polysaccharide complexes may be greatly altered by the action on only one of the components.

Holbrook: I understood Dr. Perlmann to say that the periodic acid-Schiff can be obtained in material not containing polysaccharides.

Meyer: I absolutely would agree to this statement.

Perlmann: Even if fibrinogen would contain carbohydrate, its carbohydrate content would be small and would hardly account for the phenomena which Dr. Dempsey observes.

Dempsey: It was an intense reaction.

Perlmann. The similar comment could also be made in connection with prothrombin. Moreover, the amount of prothrombin added to fibrinogen to catalyze the transformation of fibrinogen to fibrin, is exceedingly small.

Dempsey: The fibrinogen may be a glycoprotein.

Meyer: Its carbohydrate content is perhaps too low to classify as such.

Ragan: Does your fibrin film stain metachromatically?

Dempsey: No, quite the contrary. It has a strong ability to prevent metachromasia. It behaves in that respect like nucleoprotein.

Meyer: As I said before, it cannot be denied that periodic acid acts on protein. It is very difficult for me to see that the reaction would be comparable to that of carbohydrate.

Dempsey: In purified fibrin films of 10 μ thickness, the intensity of the reaction is as strong as you can observe in any tissue section containing fibrinoid. This is an intense reaction. What it is due to I don't know.

Angevine: What animal was this? Was it human placenta that you studied?

Dempsey: Human placenta, human fibrinogen

Holbrook: Dr. Angevine, don't you want to make some comment here?

Angevine: I might say when we first looked at fibrinoid, we considered it to be degenerated collagen. That was the usual conception, and direct observation added little to our knowledge of its nature.

Altshuler and I have attempted to apply some of the things that Dempsey and Wislocki (8) have done on normal tissues to certain disease processes. We were at somewhat of a disadvantage compared to the anatomists because they always have an abundance of fresh animal tissues available for study. To obtain the necessary amount of material from a variety of diseases it was necessary for us to use tissues that had been fixed and embedded in paraffin for considerable periods. All of the tissues studied were stained with H. & E., phosphotungstic acid and hematoxylin, toluidine blue and periodic acid leukofuchsin and found a certain common staining pattern to the group of tissues studied. Obviously some of the tissues had lost some of the metachromatic material so that there was considerable variation in intensity. In the early stages of most of the lesions the acid mucopolysaccharides were very evanescent. This material is difficult to visualize without considerable experience and the light source must have no obstruction with blue filters. Our published illustrations are considerably less intense than original slides since we had some difficulty with the reproduction of purple red color. As the lesions increase in age, there is apparently a considerable loss of metachromasia; the lesions also become more hyalinized or sclerotic. One, of course, cannot control these factors in studying tissues in disease unless frequent and repeated biopsies are made from available sites. All we have attempted to p[illegible] is the manner in

which this metachromatic material appears and to speculate on how it is related to the formation of fibrinoid. It was the premise of our investigation that the essential feature of fibrinoid formation is the precipitation of the acid mucopolysaccharide of the ground substance. The precipitation might be produced in a variety of ways, either by lowering tissue pH below that of the iso-electric point of tissue proteins or by the addition of an alkaline protein (on the acid side of the iso-electric point at normal body pH). So I would not think we have any disagreement with what Dr. Dempsey has said since he can control his studies better than we. As to why it happens, I am uncertain. We are probably beginning to find out now what the Hotchkiss stain does not do rather than what it does do. It probably includes many, many reactions. They will have to be re-evaluated. I am certainly not the one to do it.

Mirsky. I am not clear. That is why I ask this question. Is it your working hypothesis at the moment that fibrinoid is the same material as fibrin?

Dempsey: Depending upon the location at which it occurs, fibrin may form a mass in which is intermixed collagen fibers and the acid mucopolysaccharides of the ground substance of connective tissue. Thus, you may have fibrin as it clots from pure fibrinogen, as it clots in a blood vessel, or as it clots in a connective tissue matrix of different compositions. Consequently, you may have substances which superficially give the same microscopic appearance but which may have varying compositions due to admixtures of other materials. The only possible point of difference between what I said and what Dr. Angevine said involves the metachromatic reaction of fibrinoid and its periodic acid-Schiff reaction. He concluded that both were ascribable to the acid mucopolysaccharides. I quibble about that and say probably only the metachromasia was a valid index of the acid mucopolysaccharide. The other reaction is due to the fibrin itself.

Angevine: We cannot identify fibrin in our sections. We certainly don't find any indication there is fibrin except occasionally. As Dr. Bennett showed, it should be intermingled. You could scarcely have any reaction without some fibrin.

MacLeod: How specific is the stain for fibrin?

Angevine: Hematoxylin or phosphotungstic acid hematoxylin?

Gyorgy: How do you stain?

Angevine: Phosphotungstic acid and hematoxylin.

Fremont-Smith: How specific is that?

Angevine. Very specific. I would not wish to be more definite.

We have studied the fibrin in experimental inflammation in normal and dicumarolized rabbits and found the stain served very well to determine the quantity of fibrin present. The diminution or lack of fibrin in the treated animals gave a good base line for such an observation (9).

Meyer: The question is how specific is the method of the uptake of dye for fibrin under conditions of graded acidity and ionic strength?

Dempsey: I have not used the phosphotungstic acid and hematoxylin method for fibrin very much. We have used the Mallory and triple stains more often. Concerning its specificity, *it is* interesting that in collagen, for which it is also usually regarded as specific, one sometimes sees a single fiber which is red on one side [illegible]

Meyer: Is there any other difficulty under these conditions?

Dempsey: No, because it depends upon the method of utilizing the dye. If you use a pure basic dye and if you control the ionic environment of that dye it seems to me that the uptake of the dye is governed by the same kinds of laws which govern the uptake of any other kind of base. On the other hand, in these more complex histological stains the procedures are uncontrolled and the stain is applied so that the section is overstained. Then one differentiates until the stain comes to a point at which it looks agreeable to the investigator. So it is not the same kind of method.

Meyer: I don't know. Can we go ahead and presume some of the work which Dr. Perlmann is going to discuss? It seems to me that the uptake of dye by a substance in a complex structure is not necessarily identical with the uptake of dye by the isolated substance in solution. Some of the ionizable groups may be present as such and some in complex forms, and you would have to deal with competitive complex formations, aside from the question of adsorption. The problem of the binding of dyes and of some anionic substances by wool as studied by Steinhard shows how complex these problems are I don't know whether Dr Perlmann agrees with this.

Perlmann: I agree with Dr Meyer that these reactions are very complex. That, *I feel,* is one more reason why I would caution against making too many deductions pertaining to specificity, particularly if these reagents are being used in histochemical work

Fremont-Smith But there is now a discrepancy of interpretation and the use of two different stains If you both used the same

stains would you not be more likely to come to comparable interpretation?

Dempsey: We would get the same reactions, make the same observations.

Angevine: I think we would. Am I correct in saying that you only find this in placenta?

Dempsey: Yes, that is the only place we studied.

Angevine: We studied the placenta also and found the same thing you did except I would say that in the placenta and in the base of the gastric ulcer the material appears a little more like fibrin than in the other areas. We might be encompassing too much in our definition.

Dempsey: Maybe this word "fibrinoid" is too loosely used here and maybe it means one in—

Ragan: If you continue to feel that fibrinoid stains metachromatically you would say that the material in an H. & E. stained section of healing callus is fibrinoid.

Fremont-Smith: Fibrinoid that stains metachromatically.

Dempsey: Fibrinoid plus something else added to it. I want to argue the question Dr. Meyer raises that one cannot use stains as chemical reagents. I don't quite agree to that. If you take identical pieces of fibrin film—I am now quoting work Dr. Singer has done in our department—and study the factors which vary the uptake of a basic dye, methylene blue, by those pieces of fibrin film, you find that in order to get reproducible staining you have to control five variables. One must control the concentration of the dye. One must control the length of time that the tissue is stained and the best way to do that is to stain in dilute solution and stain to equilibrium (the point where further increase in time gives no further change in the uptake of dye). One must control the pH. One must control the ionic strength of the buffer used and one must also control the previous chemical treatment of that piece of fibrin film. If those five variables are controlled, then this piece of fibrin film will take up a measurable quantity of dye. That dye may be measured today and determined a year from today. You can repeat the experiment and come out within 2 percent to 5 percent of the same amount of dye taken up by the same weight of film. So with control of these five variables one has a reproducible uptake of dye. I cannot really see where there is any difference here from determining the uptake of any other charged substance. It is not necessarily an acid-base reaction of the dye with the solid, precipitated material but it seems to me that it must be a reaction between charged groups of the solid

protein and the charged groups of the dye. About the only difference between this and the chemical titrations of proteins is that the chemical titration is done in solution whereas this is done with protein in the solid state. If it is true that a protein in a solid state can never be analyzed chemically or its charge can never be determined, then I retire from all this. Nevertheless, I would like to state my faith that substances in the solid state can be worked with, with as much chemical precision as those in solution. However, one must determine the laws which govern their reactions.

Meyer: I doubt it. Can you compare one fibrin film with another, using all your five criteria?

Dempsey I can take a film of the same thickness and make two preparations. Suppose we make a film from purified fibrinogen. If we cut several pieces from that single film and stain them, controlling four of the variables and allowing the pH to vary, we get a reproducible curve of dye-uptake. If we use an acid dye, the curve goes in one direction. If the experiment is repeated tomorrow we get almost identical curves. We can contrast these curves with similar ones obtained from other proteins. If we apply these methods to tissue sections, we find that the basophilic substance of nuclei fail to stain below pH 4 0, whereas the basophilic substance of muscle fails to stain below pH 5.0 and that of cartilage continues to stain as low as pH 1.5. Thus by these tactics we can characterize the acidity of different microscopic structures, and our methods are reproducible. This is all I am claiming. It seems to me that here are the elements of a chemical method.

Angevine: Dr. Fremont-Smith, in answer to your previous question about staining, we did van Gieson's stains on all of our material. In the few placental tissues examined the fibrinoid material in Nitsbuch's membrane, that in the subchorial plate and that involving the degenerative chorionic villi gave the same staining reactions as fibrinoid occurring elsewhere. Phosphotungstic acid hematoxylin stains indicated that fibrin might be precipitated in considerable quantities in some areas. It would not be correlated, however, with the distribution of the fibrinoid material

Fremont-Smith Obviously this is an area where it seems possible now to come to grips and either to find out that you are in agreement or if not, be able to specify the nature of your disagreement.

Angevine: I must admit in relation to this that we have studied the placenta and gastric ulcer only incidentally to indicate that this subject is much broader than we have described in our initial studies.

stains would you not be more likely to come to comparable interpretation?

Dempsey: We would get the same reactions, make the same observations.

Angevine: I think we would. Am I correct in saying that you only find this in placenta?

Dempsey: Yes, that is the only place we studied.

Angevine: We studied the placenta also and found the same thing you did except I would say that in the placenta and in the base of the gastric ulcer the material appears a little more like fibrin than in the other areas. We might be encompassing too much in our definition.

Dempsey: Maybe this word "fibrinoid" is too loosely used here and maybe it means one in—

Ragan: If you continue to feel that fibrinoid stains metachromatically you would say that the material in an H. & E. stained section of healing callus is fibrinoid.

Fremont-Smith: Fibrinoid that stains metachromatically.

Dempsey: Fibrinoid plus something else added to it. I want to argue the question Dr. Meyer raises that one cannot use stains as chemical reagents. I don't quite agree to that. If you take identical pieces of fibrin film—I am now quoting work Dr. Singer has done in our department—and study the factors which vary the uptake of a basic dye, methylene blue, by those pieces of fibrin film, you find that in order to get reproducible staining you have to control five variables. One must control the concentration of the dye. One must control the length of time that the tissue is stained and the best way to do that is to stain in dilute solution and stain to equilibrium (the point where further increase in time gives no further change in the uptake of dye). One must control the pH. One must control the ionic strength of the buffer used and one must also control the previous chemical treatment of that piece of fibrin film. If those five variables are controlled, then this piece of fibrin film will take up a measurable quantity of dye. That dye may be measured today and determined a year from today. You can repeat the experiment and come out within 2 percent to 5 percent of the same amount of dye taken up by the same weight of film. So with control of these five variables one has a reproducible uptake of dye. I cannot really see where there is any difference here from determining the uptake of any other charged substance. It is not necessarily an acid-base reaction of the dye with the solid, precipitated material but it seems to me that it must be a reaction between charged groups of the solid

protein and the charged groups of the dye. About the only difference between this and the chemical titrations of proteins is that the chemical titration is done in solution whereas this is done with protein in the solid state. If it is true that a protein in a solid state can never be analyzed chemically or its charge can never be determined, then I retire from all this. Nevertheless, I would like to state my faith that substances in the solid state can be worked with, with as much chemical precision as those in solution. However, one must determine the laws which govern their reactions.

Meyer: I doubt it. Can you compare one fibrin film with another, using all your five criteria?

Dempsey: I can take a film of the same thickness and make two preparations. Suppose we make a film from purified fibrinogen. If we cut several pieces from that single film and stain them, controlling four of the variables and allowing the pH to vary, we get a reproducible curve of dye-uptake. If we use an acid dye, the curve goes in one direction. If the experiment is repeated tomorrow we get almost identical curves. We can contrast these curves with similar ones obtained from other proteins. If we apply these methods to tissue sections, we find that the basophilic substance of nuclei fail to stain below pH 4.0, whereas the basophilic substance of muscle fails to stain below pH 5.0 and that of cartilage continues to stain as low as pH 1.5 Thus by these tactics we can characterize the acidity of different microscopic structures, and our methods are reproducible. This is all I am claiming. It seems to me that here are the elements of a chemical method.

Angevine: Dr. Fremont-Smith, in answer to your previous question about staining, we did van Gieson's stains on all of our material. In the few placental tissues examined the fibrinoid material in Nitsbuch's membrane, that in the subchorial plate and that involving the degenerative chorionic villi gave the same staining reactions as fibrinoid occurring elsewhere. Phosphotungstic acid hematoxylin stains indicated that fibrin might be precipitated in considerable quantities in some areas It would not be correlated, however, with the distribution of the fibrinoid material.

Fremont-Smith. Obviously this is an area where it seems possible now to come to grips and either to find out that you are in agreement or if not, be able to specify the nature of your disagreement.

Angevine: I must admit in relation to this that we have studied the placenta and gastric ulcer only incidentally to indicate that this subject is much broader than we have described in our initial studies.

MacLeod: If one were seeking to identify a substance fibrin, in fibrinoid degeneration or in other areas, I wonder whether it might be possible, now that highly purified fibrinogen and fibrin can be obtained, to immunize animals with them and to prepare specific antifibrin antibody. This could then be made fluorescent and applied to the sections, or whatever else one may be studying, to find out actually whether the fibrinoid material does contain fibrin. This might be a specific test for fibrin.

Angevine: Wherever there is an injury there must be some fibrin admixed, because one cannot have an increase in tissue fluid without some fibrin. *There is a small amount of fibrin in lymph wherever there is edema. With the most minimal injury you will* have it.

Ragan: Has any other substance been overlaid on this curve with fibrin? Have you done some of the mucopolysaccharides?

Dempsey: We have not studied that point specifically. The mucopolysaccharides, depending upon their location, vary enormously in their acidity. Cartilage and the mucus of goblet cells are very strongly acid. Those of vitreous humor and the cornea are less acid. Those of the umbilical cord are still less so. It is possible to have a mucopolysaccharide which will overlap with this curve.

Ragan: Then you cannot say that this is fibrin. You cannot *say specifically?*

Dempsey: I said I did not have any evidence that it was not fibrin.

Ragan: Fibrinoid may contain fibrin plus glucoprotein.

Dempsey: That is a working hypothesis.

Meyer: I wonder whether *an increase in metachromasia* necessarily means an increase in concentration of acid mucopolysaccharides or could it mean the appearance of groups masked in normal tissue. Hasn't Sylvén postulated this?

Bennett. I have been uncertain as to whether the property of metachromasia really means fibrinoid.

Angevine: I don't think it does.

Bennett: We are concerned with the problem of whether the property of increased metachromasia means fibrinoid change or represents an initial stage in its development. Metachromasia can be obtained by injuring connective tissues *without the development* of the latticing effect and increased fibrillation that was illustrated in the figures. I am not sure we are all speaking of the same change when we refer to fibrinoid.

Angevine: We do not consider that increased metachromasia

indicates fibrinoid. We have been attempting to point out that metachromatic material is present before fibrinoid is laid down or appears. There is a previous increase in mucopolysaccharides.

Holbrook: Say that again?

Angevine: We are sure of the fact that there is always an increase in mucopolysaccharides before fibrinoid appears. The literature gives six or seven theories of how fibrinoid forms. Of that group we felt the best explanation is that there is a combination of the acid mucopolysaccharide with some protein in the tissue to form fibrinoid. Whether it is a glycoprotein or exactly what it is, I cannot answer.

Fremont-Smith: On the basis of the fact that there is metachromasia, that in your opinion is a measure of the mucopolysaccharide?

Angevine: A rough measure.

Fremont-Smith: Is that the main basis for saying mucopolysaccharide always precedes the appearance of fibrinoid? Is it agreed that it is a good basis?

Meyer: I would not say it is generally agreed. It is a little dangerous to make this statement unless it is better defined.

Dempsey: This is another example of a semantic difficulty. To the morphologists, a mucopolysaccharide is something that is metachromatic and gives a positive periodic acid-Schiff reaction. To the chemist, the word indicates an entirely different set of operations. We will have to define what we each mean by the word.

Fremont-Smith: I think it is very important because in this discussion whenever any of this group or in the field uses the word "mucopolysaccharide" they ought to say, "as defined by means of" or, "as defined chemically," because it makes all the difference in the world. This could lead to endless confusion.

Angevine: We assume this encompasses a large number of substances.

Holbrook: Did I understand you to say that metachromasia can be demonstrated prior to the development of fibrinoid?

Angevine: Oh, yes.

Holbrook: How long a period? How do you know that? Does fibrinoid always develop following metachromasia?

Angevine: No, metachromasia can be reversible.

Holbrook: Are you reasonably sure that metachromasia is step one of fibrinoid?

Angevine: That has been our working hypothesis.

Mirsky: Has there been an experimental condition where you

can produce metachromasia and have it become fibrinoid?

Angevine: Yes, we have x-rayed the chick membrane and have seen this material accumulate. A good many workers have done it also.

Mirsky: The reason I voice the question that way is to point up Dr. Holbrook's question. That would indicate that one would follow the other.

Angevine: It is not all morphological observation. It has been done under experimentally controlled conditions.

Mirsky: It is not fair to say that the fibrinoid reaction being discussed this afternoon is just a morphologically describable phenomenon and does not necessarily have any relationship to some specific chemical substitution.

Angevine: I think so.

Mirsky: I would like to hear some discussion on it.

Dempsey: I would object to that on the same ground that Dr. Szent-Gyorgyi did this morning. If morphologically recognizable there must be some chemical rationale.

Mirsky: I said specific. The point I was getting at is: Can we have a number of different chemical changes occurring in tissues, some around the vessels, some in the joint and some in the valve, each one of which will give us this appearance?

Holbrook: Not if due to mucopolysaccharide.

Dempsey: It seems to me we are down to the point where we are pretty sure there are three chemical elements that are at least suspected of being in this mass of material. One is collagen, in most of the locations. One is the ground substance of the connective tissue, which is in most locations, and the third is fibrin, which is present demonstrably in at least some of them. Here then is something that has a variable amount of three components The question is to specify quantitatively just how much of each one of the elements is in each one of the locations. Isn't that about it?

Bennett. That expresses it very well, but there is one other consideration, namely: How much degradation of these components occurs as the lesions progress?

Holbrook: Is there any further discussion about this question? Is there any more to say about that particular thing, Dr. Dempsey?

Dempsey: I shot my bolt.

Szent-Gyorgyi: Did you ever try fibrinolysin?

Dempsey: We never tried any proteolytic enzyme on this material.

Jones: It would seem to me that some such methods or things

can produce metachromasia and have it become fibrinoid?

Angevine: Yes, we have x-rayed the chick membrane and have seen this material accumulate. A good many workers have done it also.

Mirsky: The reason I voice the question that way is to point up Dr. Holbrook's question. That would indicate that one would follow the other.

Angevine: It is not all morphological observation. It has been done under experimentally controlled conditions.

Mirsky: It is not fair to say that the fibrinoid reaction being discussed this afternoon is just a morphologically describable phenomenon and does not necessarily have any relationship to some specific chemical substitution.

Angevine: I think so.

Mirsky: I would like to hear some discussion on it.

Dempsey: I would object to that on the same ground that Dr. Szent-Gyorgyi did this morning If morphologically recognizable there must be some chemical rationale.

Mirsky: I said specific. The point I was getting at is: Can we have a number of different chemical changes occurring in tissues, some around the vessels, some in the joint and some in the valve, each one of which will give us this appearance?

Holbrook: Not if due to mucopolysaccharide.

Dempsey It seems to me we are down to the point where we are pretty sure there are three chemical elements that are at least suspected of being in this mass of material. One is collagen, in most of the locations. One is the ground substance of the connective tissue, which is in most locations, and the third is fibrin, which is present demonstrably in at least some of them. Here then is something that has a variable amount of three components. The question is to specify quantitatively just how much of each one of the elements is in each one of the locations. Isn't that about it?

Bennett: That expresses it very well, but there is one other consideration, namely: How much degradation of these components occurs as the lesions progress?

Holbrook: Is there any further discussion about this question? Is there any more to say about that particular thing, Dr. Dempsey?

Dempsey: I shot my bolt.

Szent-Gyorgyi: Did you ever try fibrinolysin?

Dempsey: We never tried any proteolytic enzyme on this material.

Jones: It would seem to me that some such methods or things

other than staining characteristics might give you your clues.

Mirsky: Fibrinolysin?

Jones: Not necessarily that.

Mirsky: We were discussing that question. Would the specific proteolytic enzymes be of value in distinguishing it? It would be very difficult because to begin with, we don't know what fibrinolysin is

Mote: I don't think we know what fibrinoid is either.

Dempsey: It seems to me that it is possible to tackle the problem by isolation techniques. One could simply identify a particular lesion, isolate it and determine by standard quantitative analyses what chemical substances were present. It is simply a matter of identifying, on the one hand, the morphology of the lesion and, on the other, its chemical composition.

Meyer: The experimental setup has already been suggested as far as I can see. Dr. Angevine suggested the production of lesions in animals under dicumarol. The transformation of fibrinogen to fibrinoid takes place in these animals or takes place to some degree. This already has been done or this has not been done?

Angevine: We have not done it. I mentioned that in connection with the specificity of the fibrin stain. That might be one way to approach it. One could produce fibrinoid lesions in such animals with a very low level of fibrin. Fibrinoid lesions could be produced in blood vessels very readily. I have never seen it done in a dicumarolized animal

Mirsky: You speak of the ease with which the fibrinoid lesion can be produced. Where does it belong in our thinking? What kind of lesion is it? Is it so nonspecific in so far as response to stress or injury?

Angevine: I did not quite get the question.

Mirsky: You have made the statement several times about the ease with which the lesion can be produced. How easy? What produces the lesion?

Angevine: You can produce it by injury. If one were to pinch the skin and to section it subsequently, an increase of metachromatic material could be demonstrated. You can burn it slightly or —

Mirsky: What relationship has this got to Hans Selye's adaptation syndrome? Is it a manifestation of the so-called alarm reaction?

Angevine: I would not attempt to answer that. However, we have examined some tissue from some arthritic rats that Selye kindly sent us. Around the joint there is an increased amount of

metachromatic material. That merely indicates tissue injury. A similar lesion might be produced in several ways. I would also like to re-emphasize a statement Dr. Bennett made, that there are a variety of ways by which metachromasia can be produced.

Dempsey: If you simply inject a little acid subcutaneously you get something very similar.

Bennett: Perhaps the simplest method is the one that Dr. Angevine has referred to, traumatization—particularly traumatization where there are repeated shearing and bruising forces involved. With the exception of some of these lesions that seem to be induced by hypersensitivity mechanisms, it would seem that the forms of injury that are most likely to produce fibrinoid changes are the ones that cause extravasation of plasma into the tissues. A number of years ago, when pressed to state what I believed fibrinoid was, I expressed the thought that it was fibrin soaking of connective tissue. I still believe that this is the mechanism by which some "fibrinoid" lesions develop.

Travell: I wanted to ask whether the fibrinoid changes can be related to any other connective tissue changes such as amyloid degeneration or localized myolysis of muscles, and so forth?

Angevine: We initially restricted our investigation to fibrinoid in rheumatic diseases because the whole subject seemed so diffuse. However, it should be emphasized that these acid mucopolysaccharides are very widely distributed. It is found in cartilage and growing bone. It occurs in young fibrous connective tissue and when there is an increase in amount it may remain or be absorbed. It also occurs in association with hyalin and amyloid. Because of this there is reason to believe that hyalin and amyloid are closely related substances.

György. What is hyalin?

Angevine: That would correspond to sclerosis. A sclerosed vessel is hyalinized. I do not know what it is chemically, but morphologically it is an eosinophilic material.

Bennett: Do you think it proper to refer to hyalin as a substance or should one speak of the altered tissue as being hyalinized?

Angevine: We speak of amyloid material. I don't see why we [illegible] material or substance. There is said [illegible] yalin. [illegible] zed it does not tell you actually what the process is.

Bennett: It certainly does not, but as Dr. Angevine just said, he does not know what hyalin is and no one else knows either.

Angevine: This metachromatic material accumulates in all in-

stances before fibrinoid change, sclerosis, etc, develop.

Meyer: Do you believe it is an actual increase in quantity of all these processes or do you think it is a shift, an accumulation from the neighboring areas? Is it just the liberation of something which occurs?

Angevine. There is certainly more than one sees normally. That is in the manner we have performed our studies. We have not determined it chemically. This material accumulates in areas where it is not present normally.

Meyer Does the primary change involve the ground substance or the cells?

Angevine: Well, it would vary. Of course, in fibrosis the primary change would be a cellular one.

Meyer: I meant the first reaction toward a nonspecific trauma as you described it.

Angevine: There would be cells but serous inflammation or edema is relatively acellular, as the chief component is fluid with very few cells I don't think you can have any change in the body without some cells

Bennett: I think that what Dr. Meyer meant to ask was, do you have any notion as to whether the primary change leading to an increase in metachromasia is a cellular phenomenon or something that resides in the intercellular substances.

Angevine: There is an element of both, to evade the question The most significant thing, though, seems to be the accumulation of material in the ground substance and the cells merely accompany the reaction to a certain extent.

Fremont-Smith: What is the end point in metachromatic staining, as I understand it? When you use metachromatic stain, it is differentiated as to taste or something of that sort, is it not?

Angevine: Stained with toluidine blue for a certain period. It is very evanescent as a stain. It is not permanent. We use it for a standard length of time at a standard concentration. We do not differentiate

Fremont-Smith: I think someone in the discussion speaking of the stain did speak of it in the other way

Dempsey· That was a different stain.

Angevine: It is a different stain. We stain to a certain saturation point and stop. We don't overstain and differentiate.

Fremont-Smith So the slides are comparable

Angevine: I think so.

Holbrook. Do you know whether mucopolysaccharide or the metachromasia is wholly in the ground substance or the fibroblast? What substance does it show itself in?

Angevine. We see it only in the ground substance and in the tissue mast cells. They contain it and they are known to form heparin. *They stain very well metachromatically. I do not recall* anything else, Dr. Dempsey, do you?

Dempsey: There are occasional droplets in the macrophages.

Mote: Fibroblasts.

Holbrook: Yes.

Meyer: The inclusions which Gersh has described in some macrophages and fibroblasts are in different stages of activity. They do not stain metachromatically?

Ragan: No. I asked him about that and he said that they did not stain metachromatically.

Holbrook: The fibroblasts do not stain.

Jones: You seem to use the term "mucopolysaccharides" and "metachromatic staining" synonymously. Do you mean this?

Angevine: Mucopolysaccharides are metachromatic, and in tissues they include a large variety of substances. It is a pretty loose term chemically. I will agree with that. If there is a free acid radical present, one can demonstrate metachromasia. If this is blocked or removed, metachromasia is abolished.

Holbrook: I guess we will have to wait until tonight to ask Dr. Meyer where the mucopolysaccharides come from.

Angevine: I hope he will be able to explain that.

Meyer: I am afraid the histologists will have to answer these questions. We work with whole tissues and don't know from what part of the tissues the substances are derived.

Ragan: I would like to ask Dr. Bennett if he has used silver stains on any of these fibrinoid reticulum fibers? Do they continue through the fibrinoid?

Bennett: I have used reticulum stains occasionally. It seems to depend upon the stage of development of the lesion as to whether or not the altered fibrils are continuous. Certainly they do not continue across the lesions where there is complete disintegration of the central parts of a "rheumatic" nodule However, they may continue across some of the early lesions, for example, the Aschoff *nodules such as I demonstrated earlier in the discussion*

Dempsey: Is that possibly caused by the density and consistency of the matrix of the lesion? For example, I believe a silver stain doesn't ordinarily demonstrate any argyrophilic fibers in cartilage. However, if the matrix of the cartilage is removed to expose the entrapped meshwork of collagenous tissue, the reticulum can then be stained by either the aniline blue or the silver methods. I have a feeling here that they are just covered up and cannot be reached.

Bennett: That is true to a certain extent, but in some of the lesions which usually are regarded as showing fibrinoid degeneration we have complete tissue breakdown You can see it with any stain you use: complete disorganization of fibrillar pattern and loss of cells. There is a stage in the development of such lesions when the fibrillar pattern is preserved and can be seen by applying appropriate stains, but in a further stage of development of this process, fragmentation and disorganization occurs and eventually liquefaction of the material results. Has that been your experience, Dr. Angevine?

Angevine: That is definitely true. We don't see that in the early lesions. We were concerned mostly with older lesions, and it always occurred

Bennett: There may be an appropriate place to use all of the common terms relating to fibrinoid change. It might be appro- [illegible]

be a succession of tissue changes ending in complete disintegration

Holbrook: In that chain of events is the fibroblast late to show any changes?

Bennett: It is my thought that the fibroblast is affected quite early, perhaps not in the initial accumulation of metachromatic substances but in the stage in which we see the fibrin or fibrin-like deposit. At that time, almost invariably, there is swelling and rounding of the fibroblasts. From this stage on, the morphologic appearances of the fibroblast change and eventually there is a certain degree of palisading.

Dempsey. Those palisades, as I remember from your figures were big, fat cells [illegible] Are they comparable t[illegible]

Bennett: Yes. I [illegible] palisaded cells are clearly macrophages but the association of other cells with intercellular substances clearly identifies a fair number, perhaps a majority, as proliferating fibroblasts.

Jones. Dr. Angevine, do the changes you listed here—fibrosis, sclerosis, and so forth—represent general types of connective tissue changes?

Angevine: That is correct.

Jones: You spoke of the difficulty of explaining to students what hyaline change meant. Is there any possibility of determining the fundamental mechanism which may be responsible for an alteration of the connective tissue from the normal, by varying

the type of stress or the type of noxious agent? We don't know much about that, do we?

Angevine: We certainly don't and, in fact, this conception does not help much, but it seems to be a point at which we may gain further information that will eventually simplify the problem. If we consider all of these properties as separate entities, it suggests that there is no relationship. There may well be a considerable overlap in some of these things. Take amyloid, for instance. Some amyloid gives a metachromatic staining and some does not, so obviously there is something present in one form that is not in another.

Jones: The thing that worries me is that very often we arbitrarily base our classifications on some characteristic or some method. If we type bacteria, for example, we use some chemical constituent for our classification. This constituent may not necessarily be connected with a significant biological phenomenon on which we could base a logical classification. I was just wondering whether some fresh thinking on classification based on other than visual descriptive data including staining characteristics might not be called for. In other words, the characteristics that we are using now may not be the determining features of tissue change at all.

Mote. You are at the jumping off stage in getting to the matter of definition of just what these things are.

Jones: I am sure of that.

Mote: What is their chemical relationship to each other? Are they all the same phenomena or are we actually looking at, measuring different kinds of phenomena? Until you get some totals to get at that differentiation we are going to continue to have difficulty with semantics and classification.

Jones. I think that is true, but that, in my opinion, would not alter the fact that we need some freshness in methods and approach.

Fremont-Smith· I believe the thing which is most evident in this whole day's discussion so far, is that we are not making rapid progress by these methods. It may be that they are the best we have and that we may have to plug along with them. However, it is to be hoped that we can find a fresh approach which would be much more likely to pull the things together. What that approach is to be I don't know.

I have to ask two questions. The first is: Is there any relationship between cirrhoid and fibrinoid chemically and otherwise? Secondly: Is there anything in the callus of healing fracture that resembles fibrinoid?

Gyorgy Before the first question is answered, may I ask the pathologists whether they have found fibrinoid in parenchymatous organs like the liver and kidney which have practically no ground substance? If I am not mistaken, fibrous proliferation tissue occurs in liver.

Angevine: I have never seen it in the liver. In the medulla of the kidney there is a large amount of metachromatic material and you can see fibrinoid there.

Gyorgy: You don't find it in the liver?

Angevine I don't.

Bennett I don't.

Gyorgy: You have fibrous proliferation?

Angevine: No

Gyorgy. That excludes it.

Fremont-Smith That is why I mentioned the moiety, is cirrhoid metachromatic?

Gyorgy No.

Fremont-Smith: What about callus?

Angevine: Yes.

Fremont-Smith You see fibrinoid in callus?

Angevine. Yes.

Fremont-Smith You see it in all healing wounds or generally in healing wounds cut through the skin?

Angevine: My impression is you would see it in all.

Fremont-Smith: Was that listed in our group of things? I don't remember

Ragan. It is not our impression that you see fibrinoid You see metachromasia You see fibrin but not fibrinoid.

Fremont-Smith. It is the same problem again.

Ragan: This does not stain as fibrinoid.

Fremont-Smith If you do see it in healing wounds, then haven't you an experimental method there which could be used to advantage to explore this?

Angevine: I indicated this morning that studying healing wounds and fractures would be a good method.

Fremont-Smith: I may have missed that

Angevine In my concluding statement I said that I thought it would be a better way to investigate the processes than just in normal growing and developing organs.

Holbrook Metachromasia always precedes fibrinoid. Then you may not see it in healing wounds, the fibrinoid phase, but only the metachromasia phase.

Did you say, Dr. Ragan, that metachromasia was present in healing wounds?

Ragan: Yes. If the callus of a healing fracture is followed from day to day, a period of metachromasia is seen.

Holbrook: Not fibrinoid?

Ragan: We cannot see fibrinoid.

Angevine. I think we can reconcile this difference. The wounds that Dr. Ragan describes are freshly made wounds, not complicated, as in the making of an incision in the skin. You would not see it there, but if you observe a type of wound such as we see at autopsy, that is, a wound complicated by many things, on the surface of such lesions we see some fibrin. That would correspond to the base of an ulcer. In a noncomplicated healing wound it is not present, but in other types I suspect it would be.

Fremont-Smith: Uncomplicated healing fracture?

Angevine: Yes, I don't think you would see it there. I don't recall actually looking for it or having seen it.

Bennett: In healing fractures, especially those that have not been properly immobilized, there is an accumulation of plasma and other blood elements in the tissues resulting in fibrin deposition. I am not certain that there is "fibrinoid."

Fremont-Smith: Could you not produce it in some quantity in such a lesion where you get quite a callus formation? I am thinking in terms of chemical extraction in the area where you have it highly concentrated. This might provide a basis.

Ragan: Our rabbits are not splinted. They just stand on their broken legs and have good callus and very good metachromasia. We don't see this lattice-like appearing fibrinoid.

Angevine: You don't have to have any appearing.

Gyorgy: You may have fibrinoid.

Angevine. I would like to report on this at a later date. We have just finished a study on healing fractures, but I do not recall looking for fibrinoid material I think that would be very worthwhile looking into, if Dr. Ragan did not see it, as he said.

Ragan: Yes. In the callus of a fracture, studied day by day, a period of metachromasia is seen.

Dempsey: There wasn't any argument 20, 30, or 40 years ago about the presence of fibrinoid in different regions It all looked alike. Since a variety of methods have been applied, it begins to appear that what was formerly regarded as fibrinoid has characteristically different reactions in different locations. What formerly was classified as an entity now turns out to have several subspecies.

I would like to debate your statement, Dr. Fremont-Smith, that these methods are not getting anywhere. It seems to me they are.

This represents merely the initial confusion necessary before a new set of facts get thoroughly sorted out.

Fremont-Smith: I don't mean they are not getting anywhere, but the acceleration of progress that one would like to see does not seem to be taking place within this particular area with these particular methods. It would seem to me that we need a fresh language and a fresh approach as far as the question of fibrinoid or fibrinoids is concerned because there is obviously quite a lot of confusion in the use of the term and how you define it. It appears to me that a meeting of this kind would make this quite clear.

Bennett: I am inclined to agree with that point of view. There are certain pathologic entities or, better yet, lesions that have significance now, just as they did when they were first described. Take the Aschoff nodule, for example. This lesion is as nearly disease-specific as any a morphologist observes under the microscope. The subcutaneous nodule occurring in patients with rheumatoid arthritis is also highly specific. These lesions in their total composition are very different, yet both are referred to as having *fibrinoid* qualities. Perhaps there is no good reason to continue to use the term "fibrinoid change," at least with the connotation that is usually held for the term. An alternative use of the word fibrinoid would be to designate, in a purel[illegible] anner, any change in tissue havin[illegible]

In the study of fib[illegible] might resort to the experiments [illegible] forms of tissue injury and, using unfixed or living tissues, attempt to follow the changes in and the movements of the ground substances that are contained within the fibrillar framework of connective tissue.

Holbrook Do you have something, Dr. Ragan?

Ragan: I wonder if the pink staining material in a toluidine preparation would be considered to be fibrinoid.

Angevine I should not think so That is what you get in a five-day fracture. The purplish red material would represent mucopolysaccharides or metachromatic material.

Gyorgy I would not call it fibrinoid.

Angevine With hematoxylin-eosin stain it would not look like what we called fibrinoid I think we have looked at enough of these sections to be able to differentiate one from the other. I think *there is a certain definite characteristic, if anything were fibrinoid* we could see it. I think this substance has a little more body to it.

Gyorgy. There is magic in this statement. Can you explain why it is not?

Angevine I don't see it.

Gyorgy What don't you see?

Angevine: This is fairly homogeneous material. The fibrinoid—

Gyorgy: It has to be granular?

Angevine: Diffuse and granular, much more.

Fremont-Smith: That is an aspect that has not yet come into the definition.

György: Granular as being an essential character.

Angevine: I think that is true in these metachromatic stains.

Fremont-Smith: Would you say in general that they must always be granular to be called fibrinoid? Because, if so, we have added another dimension which would be very helpful.

Angevine: Yes; would you not agree, Dr. Bennett, that there is a granular characteristic?

Bennett: There is an acquisition of granular properties as the change becomes more marked in about the third stage of development.

Ragan. Would you say it should be acellular, too?

Angevine: Fibrinoid may or may not be acellular. There can be fibrin in it and be cells in it, depending upon —

Fremont-Smith: It only has to be granular in one stage, Dr. Bennett said. You said it must always be granular.

György: That is right.

Fremont-Smith: I am trying to push, do we or don't we mean that and who means which?

Angevine. I think that it is granular.

Gyorgy: Always granular, not in this third stage?

Fremont-Smith. I thought it was granular in the third stage I am not trying to bring out the discrepancy. I think it is worth pushing a bit.

Angevine. It certainly has lost its homogeneity and has some granular characteristics in all stages

Fremont-Smith. Would we not agree that each person here probably uses a somewhat different definition for fibrinoid and probably also adds certain definitions which he does not express? For instance, it took a little while, in fact it took this discussion about granularity and Dr. Gyorgy's pushing you, sir, to make you say "granular" which had not been said before and which apparently you use unconsciously as a defining, measuring rod. I wonder whether in this situation we have not a number of conscious or unconscious factors. A person may say, "I don't see it there," and then you push him and then he says, "Well, in my viewpoint it must have crisscrosses in it or something."

Angevine: I think that is definitely true. When one looks at

something routinely one doesn't pay as much attention to it as when seen for the first time.

György: Why did Dr. Bennett say that in the third stage there are granular properties, therefore distinguishing the first stage, second stage, and third stage?

Bennett: In the first stage of development of the lesion as I conceive of it, there is an accumulation of something in the ground substance which disperses the collagen fibers. In the second stage there is an increased prominence or swelling of the collagen fibrils. In the third stage of development there is visible granularity of the substance that forms around the collagen fibrils. I have in mind the Aschoff nodule as exemplifying these stages in progression of fibrinoid change.

Gyorgy: The metachromasia has no place in the definition?

Bennett Increased metachromasia appears early, probably in the first stage of the lesion Would you agree with this concept of sequences, Dr. Angevine?

Angevine. That is the way we feel about it.

Mote. In the very acute fulminating rheumatic fever cases we saw, we certainly had some peculiar looking Aschoff bodies, what we call swelling, and in some of it, there was not so much granularity

Holbrook After all, we are talking about a name "fibrinoid," which is largely by definition, isn't it?

Angevine: That is correct.

Gyorgy If you can define it.

Fremont-Smith: By a series of different . . .

Gyorgy. Different deductions.

Holbrook: Which is what you define it. That has no clearcut reality, yet as a something you can weigh, measure and count.

Jones: People write books about it.

Mirsky. Can they agree on fibrinoid by some cues they see which have not been transmitted to us?

Holbrook: What constitutes fibrinoid is still a matter of definition to many.

Fremont-Smith: Have we any assurance that they agree? I know, and Dr. Gyorgy will remember that in the Liver Injury Conference we had a Subcommittee on Cirrhosis of the Liver in which we had a series of sections that were submitted to quite a number of pathologists for their opinions In noting the degrees of agreement and disagreement I may say that the degree of disagreement is what stood out I wonder if there is any evidence that everyone in this group would agree as to which sections out of any five were fibrinoid.

Angevine: It is the same as in the diagnosis of malignancy. If you examine a section that is just on the borderline, one way or another, the diagnosis will all depend upon the criteria you set up for malignancy based on a good many years of experience. I suspect that if you gave Dr. Bennett and me five sections containing fibrinoid graded as 1 to 5 plus in amount and asked us to give a quantitative estimate on them, we would probably agree on the slides with minimum and maximum quantities, whereas we might differ on those in between depending upon the criteria each of us might use for grading. I don't believe we would have any disagreement on what fibrinoid is.

Fremont-Smith: Do you think that would be pretty generally true among all pathologists?

Angevine: I think so.

Holbrook: I would like to ask one more question. I would like to get it clear whether you agree, in so far as you can tell by the methods we have of measuring fibrinoid, that fibroplasia is not early at fault.

Angevine. I don't think the fibroplasia is at fault at all.

Holbrook: That is what I wanted to find out. I don't know how anyone could have had a more lengthy discussion about the subject which Dr. Meyer is about to present to us. We have been talking about mucopolysaccharides since early morning and are just now to learn something about them.

REFERENCES

1 ALTSHULER, C H and ANGEVINE, D M Histochemical studies on the pathogenesis of fibrinoid *Am J Path* 25, *1061 (1949)*
2 NEUMANN, E Die Picrocarminfarbung und ihre Anwendung auf die Entzundungslehre *Arch f mikr Anat* 18, *130 (1880)*
3 KLINGE, F Der Rheumatismus, pathologischanatomische und experimentell-pathologische Tatsachen und ihre Auswertung fur das ärztliche Rheumaproblem *Ergebn d allg Path u path Anat* 24, *1 (1933)*
4 GALE, J C Electron microscopic studies of collagen from normal and diseased tissues *Am J Path* 26, *707 (1950)*
5 SCHMITT, F O, and GROSS, J Further progress in the electron microscopy of collagen *J Am Leather Chemists' Assoc* 43, *658 (1948)*
6 BENNETT, G A, ZELLER, J W, and BAUER, W Subcutaneous nodules of rheumatoid arthritis and rheumatic fever *Arch Path* 30, *70 (1940)*
7 SINGER, M, and WISLOCKI, G B The affinity of syncytium, *fibrin and fibrinoid of the human placenta for acid and basic dyes* under controlled conditions of staining *Anat Rec* 102, *175 (1948)*
8 WISLOCKI, G B, BUNTING, H, and DEMPSEY, E W Metachromasia in mammalian tissues and its relationship to mucopolysaccharides *Am J Anat* 81, *1 (1947)*
9 THUERER, G R, and ANGEVINE, D M Influence of dicumarol on streptococcic infection in rabbits *Arch Path* 48, *274 (1949)*

CHEMISTRY OF CONNECTIVE TISSUE, POLYSACCHARIDES

KARL MEYER
Department of Medicine,
College of Physicians and Surgeons, Columbia University

I WILL try to report to you on some of the work we have been doing without going into too great detail and at the same time tell you about some of the problems we are facing.

The substances which we call mucopolysaccharides of the mesodermal tissues are assumed to be present in the ground substance on the basis of histological data, because we have no specific extraction procedure for the ground substances. We have to work with large quantities of more or less mixed tissues which we get from the slaughterhouse or from the operating room. Which of the substances come from the ground substances, which from the cells and which are components of the fibrillar elements and in what proportions, we don't know. From chemical analysis of collagen or elastin it appears that the fibrils proper are free of carbohydrate.

At present
connective tis
of hyaline ca
3) chondroitin sulfate B which we first isolated from skin, 4) chondroitin sulfate C, 5) the sulfate ester of the substantia propria of the cornea which we believe to be a monosulfate ester of hyaluronic acid.

Hyaluronic acid has been isolated from vitreous humor, umbilical cord, synovial fluid, and some mesodermal tumors from skin and from cockscomb as recently reported (1). Of great importance was the finding of hyaluronic acid in group A and group C hemolytic streptococci, in Heidelberger's laboratory. Chemically hyaluronic acid is a polymer of a disaccharide composed of equimolar portions of N-acetyl glucosamine and glucuronic acid with the reducing group on the acetyl glucosamine moiety. This conclusion is based on analysis and the increase in the color reaction
yaluronidase a
ields up to 70
of hyaluronic

acid. When this fraction is subjected to the action of pneumococcal and streptococcal enzyme, the reducing sugar increases further to what was thought to be the constituent monosaccharides, N-acetylglucosamine and glucuronic acid. From chromatographic analysis and paper chromatography this product is a disaccharide of yet unknown nature. Dr. M. Rapport in our laboratory recently has thoroughly analyzed the hydrolysis products of hyaluronic acid. It appears from his studies that the end product of the action of testicular hyaluronidase may be a higher unit than a disaccharide.

We assume that the bacterial enzymes contain a specific β-glucuronidase, since the rotation of the isolated disaccharide still is negative and the known β-glucuronidase of the liver and spleen,

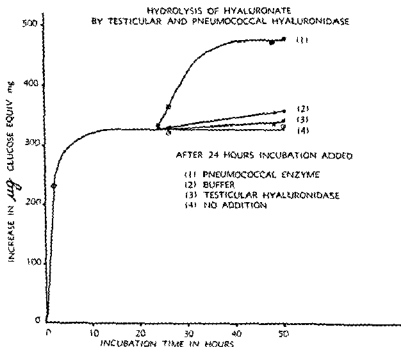


FIGURE 18

which hydrolyze such detoxification products as borneol glucuronide, pregnandial glucuronide, etc., do not act on either hyaluronic acid or its hydrolysis product obtained with testicular hyaluronidase. Figure 18 illustrates the increase in reducing sugar with testicular hyaluronidase followed by a pneumococcal enzyme after the testicular enzyme has come to a standstill.

Szent-Gyorgyi: What makes the first jump?

Meyer: The first jump is the large amount of testicular hyaluronidase.

Szent-Gyorgyi: At zero.

Meyer: No. 3, 3 and 4, zero time, it is incubation time.

Szent-Gyorgyi: At zero time what is the first?

Meyer: Figure 19 shows the hydrolysis of the hydrolysis product of testicular enzyme by both pneumococcal and a streptococcal enzyme.

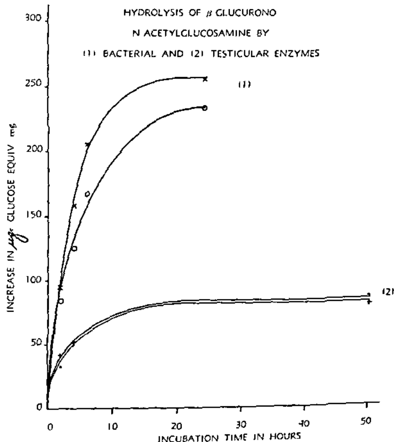


FIGURE 19

The exact molecular weight of hyaluronic acid is not known, but it is probably very high. As in so many other naturally occurring high polymers, hyaluronic acid is polydisperse. In fluids like synovial fluid it is not bound to protein but occurs as easily dissociated salt, statements in the literature to the contrary notwithstanding. The viscosity of the native fluid is at least ten times greater than that of the acid isolated from it. It appears that hyaluronic acid contains labile bonds which are broken by the procedures of isolation. The nature of these bonds is not accurately known. We proposed some time ago that they are anhydride linkages, based on a deficit in ash of about 20 percent, on a similar deficit in the amount of serum protein precipitated, and on the formation of amide N beside NH_4-N on treatment of the anhydrous acid with methylalcoholic NH_3.

The second substance I referred to, the chondroitin sulfate of hyaline cartilage can be extracted from cartilage powder by concentrated salt solution, especially $CaCl_2$. As with hyaluronic acid, this indicates that we are not dealing with a covalent linkage to protein, but to a polar or salt linkage. The molecular weight of chondroitin sulfate has been estimated by Blix and Snellman (2) from the streaming double refraction as between 200,000 and 300,000. K. H. Meyer has assigned a structural formula to this compound According to some work on the stability of the sulfate group by Dr. Rapport (3), the conclusions of the Swiss workers appear not to be justified. Chondroitin sulfate is hydrolyzed by testicular hyaluronidase, which would indicate a close similarity

TABLE I

Occurrence of Mucopolysaccharides in Mesenchymal Tissues

Group	Tissue	Hyaluronic Acid	ChS-A $(a)_D = -80°$	ChS-B $(a)_D = 50°$	ChS C $(a)_D = 20°$
I	Vitreous Humor	+	—	—	—
	Synovial Fluid	+	—	—	—
	Mesothelioma	+	—	—	—
II	Hyaline Cartilage	—	+	—	+ (?)
III	Heart Valves	—	—	+	+
	Tendon (pig and calf)	±	—	+	+
	Aorta	—	—	+	+
IV	Skin (pig and calf)	+	—	+	—
	Umbilical cord	+	—	—	+

of the hexosaminidic linkage of this compound with that of hyaluronic acid. The amino sugar of chondroitin sulfuric acid, however, is N-acetyl galactosamine with a sulfate group probably in the C_6 position.

The third and fourth mucopolysaccharide sulfate esters of the ground substance, chondroitin sulfate B and C of Table I, had better be discussed together. Both contain acetyl hexosamine, uronic acid and sulfate in equimolar concentrations. From both compounds, the amino sugar on isolation proved to be d-galactosamine, that is, they are isomeric with chondroitin sulfate of hyaline cartilage. Chondroitin sulfate B has an $[\alpha]_D$ of $-50°$, is precipitated in our fractionation scheme at an alcohol concentration of about 20 percent, and is resistant to testicular hyaluronidase. Chondroitin sulfate C has an $[\alpha]_D$ of $-20°$, is precipitated at 50 percent alcohol concentration, and is hydrolyzed by testicular hyaluronidase. Both B and C occur bound to protein which electrophoretically at a pH of 8.5 has a mobility of about 8.1 x 10^{-5}. The protein compound or mucoid contains about 35 to 40 percent carbohydrate. Free chondroitin sulfate at that pH has a mobility of about 11. The protein or proteins to which the sulfate ester are bound are distinct from collagen since they contain tyrosine and tryptophan. As to the occurrence of chondroitin sulfate B and C, B was found without C in skin, both were found occurring together in tendon, heart valves, and aorta. It may be highly significant that in umbilical cord we could not detect any B, while this embryonal tissue yielded large quantities of hyaluronic acid and chondroitin sulfate C.

We suspect that the absence of chondroitin sulfate B in umbilical cord may be related to the absence of a basement membrane in embryonal skin and the increase in thickness of the basement membrane with age in skin and in the arteries as reported by *Gersh* and Catchpole (4). These authors note further the extractability of the material giving the Hotchkiss stain in embryonic tissue and in certain pathological conditions such as scurvy. We note that the sulfate esters of connective tissue such as *skin, tendon,* and heart valves, are not extracted by neutral salts or phenol, while the sulphate ester of umbilical cord is extracted by salt solutions.

I think I had better close the descriptive part. I have jotted down a few problems. There are obviously a great number of purely chemical problems, such as the structure of the different mucopolysaccharides. One of the big problems is the histological identification of the different compounds and their origins. Then there is the question whether some of the protein compounds are

antigenic. What is the relationship of group A hemolytic streptococci to the ground substances, especially as they relate to rheumatic fever? Is only hyaluronic acid, which apparently is not antigenic, the only mucopolysaccharide elaborated by these organisms? The chondroitin sulfates B and C are bound to protein as I mentioned before and may be expected to be antigenic The protein, by the way, is split off by alkali.

MacLeod: Does that not ruin it?

Meyer: That is the only method we know at present. There is naturally the question whether any or all of these substances are involved in rheumatic processes and, if so, how? We cannot answer these questions, although there is circumstantial evidence, as Dr. Ragan and I have pointed out, linking some of these substances to rheumatic disease. I also want to mention here the studies of Altshuler and Angevine (5) and those of Gersh (4) which we were told about here.

Even if we accept that in rheumatic, as well as in other inflammatory reactions, there are changes involving the mucopolysaccharides of the ground substance, are these changes brought about by a process involving the ground substances directly or are they the results of an attack on the cellular elements? What is the normal metabolism of the fibroblasts which we assume to produce all elements of the ground substances and how are they controlled?

Holbrook You told us, Dr. Meyer, that the −20 fraction with the protein linkage is the substance responsible for the metachromasia. Did I understand correctly?

Meyer. Both sulfate esters would be responsible for metachromatic staining. These sulfate esters can be expected to give a stronger metachromasia than hyaluronic acid which itself, however, does stain metachromatically when highly polymerized. The depolymerized acid does stain orthochromatically. Both synovial fluid and vitreous humor have been reported to stain metachromatically. These fluids contain no demonstrable quantities of sulfate esters We fractionated very carefully synovial fluid of rheumatoid arthritis patients in order to detect the presence of sulfate esters but found none.

Holbrook: So there was no -20° sulfate fraction?

Meyer. No. The metachromasia in the loose connective tissue, however, would seem to me to be due to sulfate esters since there is so little, if any, hyaluronic acid

Holbrook I am trying to tie together what we talked about this afternoon. If the -20° fraction with the protein linkage is responsible for the metachromasia, in what we are talking about

here of fibrinoid, you intimated that this substance comes from the fibroblasts.

Meyer: Presumably both hyaluronic acid and the sulfate esters are produced by fibroblasts, but this has not been proven.

Holbrook: Yet this afternoon all the pathologists agreed they had never seen a fibroblast show metachromasia.

Meyer: This is apparently so.

Angevine: That does not mean it does not elaborate it.

Meyer: This may be a matter of concentration or of chemical linkages. Sylvén has said that normal skin gives no metachromasia unless you injure it. We have isolated from both pig and calf skin hyaluronic acid and chondroitin sulfate B. If Sylvén is right, which I don't know, then we have to assume that the sulfate group is not free.

Dempsey: There are a great many mast cells in many of these tissues Sylvén would indict them as the source of the sulfate containing metachromatic material which might get into the chemical extract, and which he therefore excluded from it.

Meyer: In the beginning I said that the chemist cannot decide *which cells are the sources of the substances he isolates. Nor* do I know about the quantitative aspects of these reactions, that is, what concentration in a given area is necessary to show metachromasia.

Dempsey: I don't think there are insurmountable difficulties about the quantity of hyaluronic acid necessary for metachromasia because, if I understood you correctly, you said that a one percent solution of hyaluronic acid dried on a slide and stained with toluidine blue, is metachromatic.

Meyer: Enzymatic depolymerization abolishes the metachromasia.

Dempsey: If you cut a section through the vitreous humor and mount it on a slide, it is also dried. I should think there would be about as much solid there as would occur from drying a drop of one percent solution.

Meyer: The concentration depends on the source of the vitreous humor. The concentration is very small. About 30 percent of the total organic matter is hyaluronic acid, but this is only *a fraction* of one percent. For the human eye it is about 1/20 of one percent

Dempsey: The point is that water is removed from tissues by dehydration during the preparation of sections Consequently, the concentration of any solid is vastly higher than it was during the hydrated state.

You said it was 30 percent of the dry weight?

Meyer: Thirty percent of the organic material.

Dempsey: My feeling would be there is a considerable amount of collapse.

Meyer: That is possible.

Mirsky. Even with that you are dealing with .03 percent.

Dempsey: I am lost in the mathematics somewhere so I won't belabor the point any more.

Meyer: It is possible, assuming that the vitreous humor is metachromatic and so is the synovial.

Dempsey: I wanted to ask another question about that. You indicated, if I remember correctly, that the viscosity of the natural synovial fluid and of the vitreous humor is considerably higher than that even of the best extract.

Moreover, if you dry a film of your extract upon a slide it is metachromatic. If you depolymerize with hyaluronidase, material diffuses into the supernatant and the metachromasia disappears Is it not possible then, that in the native state where there is a still larger molecule than you have in the extract, that the hyaluronic acid is less diffusible than in the model experiment?

Meyer. That is very possible I believe that that is definitely so especially after fixation.

Dempsey I am very much interested in this because Sylvén hopped all over me when he was here about the idea that a sulfate free substance might exhibit metachromasia. He simply refuses to consider the possibility that metachromasia might be caused by something other than a sulfate ester.

Meyer. The problem of solubility and diffusibility definitely enters the picture. We have encountered a hyaluronic acid containing cyst, the content of which was not soluble in saline. The cyst was from a malignant tumor of the bone. The consistency of the material was that of dried up agar. It contained a high concentration of hyaluronic acid. The material swelled up and it would have taken a long time to extract hyaluronic acid with saline. Still the enzyme depolymerized it almost explosively In the nucleus pulposus we probably deal with a similar situation. However, we have not isolated hyaluronic acid from it.

MacLeod: Sulfate-free hyaluronic acid does give metachromasia?

Meyer: As isolated from vitreous humor, yes.

Ragan: Don't you have to qualify the statement by saying if it is highly polymerized, and not depolymerized by hyaluronidase?

Meyer: Yes, it has to be.

MacLeod If you isolate hyaluronic acid from whatever source,

here of fibrinoid, you int
the fibroblasts.

Meyer: Presumably bot
are produced by fibroblast

Holbrook: Yet this aft
had never seen a fibroblast

Meyer: This is apparent

Angevine: That does nc

Meyer: This may be a
linkages. Sylvén has said t
unless you injure it. We h
hyaluronic acid and chor
which I don't know, then
is not free.

Dempsey: There are a
tissues. Sylvén would inc
containing metachromati
chemical extract, and wh

Meyer: In the beginnin
which cells are the sourcc
I know about the quantit
what concentration in a
chromasia.

Dempsey: I don't thin
about the quantity of hy
masia because, if I unders
percent solution of hyalu
with toluidine blue, is me

Meyer: Enzymatic dep
masia.

Dempsey: If you cut a
mount it on a slide, it is a
about as much solid there
one percent solution.

Meyer: The concentrat
humor. The concentratio
total organic matter is hy
of one percent. For the hu

Dempsey. The point is
dehydration during the p
the concentration of any
the hydrated state.

You said it was 30 perce

thing to be desired. It is a technical problem. The standard method originally described by Holmgren and Wilander (8) and by Sylvén (9), and used by us, involves fixing tissue in basic lead acetate. If this method is used, little or no metachromasia can be seen in normal subcutaneous connective tissue. If, however, a fresh, unfixed tissue is cut by frozen sectioning, the connective tissue matrix is metachromatic. Consequently, I think the technical method which has been used most, actually destroys much of the metachromasia.

Holbrook: Then what has that to do with it?

Mirsky: Suppose you did a fresh tissue, how are you going to know it is going to be prefibrinoid or not?

Dempsey: If it is present?

Mirsky: How can you tell if it is brilliant? Are you going to use some more cues?

Holbrook It is hard for me to understand.

Dempsey You may have substances in connective tissue which in normal amount do not indicate any pathological change but in increased amounts may so indicate.

Holbrook: But a trace of it does indicate, even a trace of this —20.

Fremont-Smith Are we talking about a normal amount of fibrinoid? Then how about that if there is a normal amount of metachromasia, and if that is an indication of fibrinoid

Mirsky All tissues are going to be fibrinoid

Fremont-Smith: Why not talk about the normal amount which has to be increased? We only call it "fibrinoid" when it is present in pathological amounts. Is that really a conclusion?

Dempsey. There is more to it.

Fremont-Smith: I know it is heresy.

Mirsky: It is going to be.

Gyorgy. Can we table it? We don't get too far with this fibrinoid as far as I can see.

Mirsky Is there a greater incidence of these morphologic changes that are associated with some kinds of tissue damage, in tissues that have the greatest amounts of—

Meyer. Metachromasia?

Mirsky I did not use the sulfated polysaccharides.

Meyer. The pathologists may have to answer that.

Ragan. I think he has isolated it in three tissues. You cannot go beyond that, can you, Dr. Meyer?

Meyer. We know very little about the quantitative relationship. The concentration of sulfated esters per gram of wet weight is

probably highest in skin and heart valves.

Mirsky: The quantity of metachromasia can apparently be directly correlated with the quantity of the sulfated polysaccharides. If that is so then you would anticipate that you are always going to get metachromasia where you get fibrinoid change, and you can talk about it as a precursor of fibrinoid change because it is going to be there anyhow.

Meyer: The question is apparently a question of the quantity, a quantitative question.

Angevine. All we have said is, there is no question but that you will see mucopolysaccharides in tissues many times in which there is no fibrinoid. On the other hand, when there is fibrinoid, and we assume it to be indicative of a pathological change, there is certainly a change in the tissues. That is always so when there is fibrinoid present. Nobody will deny this, even if they merely wish an argument.

Fremont-Smith: Touché.

Angevine: We see increase in mucopolysaccharides under these conditions. I think Dr. Dempsey will agree with that. I would like to reiterate the fact that we have not surveyed tissues of the body. Dempsey and Wislocki made the general survey and we have merely applied their techniques to diseases in which there was fibrinoid. We have always studied pathological lesions, so our approach has been a bit different, and probably some of these questions cannot be answered. Certainly on the basis of our work I think it would be a mistake to try to do so because it might lead to certain misconceptions.

Bennett: It was suggested earlier that unfixed tissues should be used for a comparative study of normal intercellular substances and intercellular materials showing fibrinoid change. This might be the best method for morphologic study.

Angevine: That would be probably the best way to approach it.

MacLeod: Does the hyaluronidase from sources other than testes cause depolymerization of the chondroitin sulfuric acid ester?

Meyer: The enzyme prepared from leech, pneumococci, and streptococci did not do it. Extracts prepared from Lister A strain of Cl. Welchii did split chondroitin sulfate, but this organism can hydrolyze everything or one can train them to do everything.

Gyorgy: Dr. Meyer, antihyaluronidase is one of the most constant findings in rheumatic fever of especially high titer. If I understood you well, you were unable to prove the antigenicity of hyaluronic acid.

Meyer: That is true.

Gyorgy· How do you explain the antihyaluronidase?

Meyer: The antibody is against the enzyme, not against the polysaccharide

Gyorgy. Why do you have that?

Meyer: Because the enzyme protein is antigenic.

Gyorgy: To protein?

Jones: There are two strains of streptococci in which hyaluronidase predominates as opposed to hyaluronic acid and which have never been associated with rheumatic fever. All of group A strains that are associated with rheumatic fever have a capsule with a very high hyaluronic acid content

Meyer Group C, too

Gyorgy. They still have

Meyer Hyaluronic acid.

Ragan There is an odd thing about the group A strain. They produce hyaluronic acid and hyaluronidase. With aging of the culture, proportionately more hyaluronidase is produced.

Jones. All the contentions have been that it is a quantitative thing, *not* that there is no hyaluronidase.

Meyer· Crawley has said they are mutally exclusive.

Jones: I don't think that is true I think most people don't agree with that.

Ragan What is produced depends primarily upon the age of the culture.

Jones: That is what I want to know

Holbrook: This –20 protein line, have you ever had enough of that to see whether or not the sulfate was antigenic?

Meyer: No We hope to do that and at the same time test the organ specificity of the ground substance. We plan to prepare the mucoid from pig tendon, inject it into rabbits and reinject the rabbit blood into the pig We might obtain that way an organ-specific serum similar to the nephrotoxic serum We don't know whether the ground substances are organ-specific. Do you know any literature on that subject besides the paper by Cavelti (10)?

Holbrook: Cavelti has published a whole series of articles on antibodies, as you know

Gyorgy The partially depolymerized hyaluronic acid in rheumatoid arthritis is a very important finding Dr Meyer, do you have any explanation for it?

Meyer· There are two possibilities. The polysaccharide is either enzymatically depolymerized or the synovial cells produce a less highly polymerized, an immature, compound, so to speak There is no evidence that in arthritis the acid is chemically any different

from the normal. We favor the theory of the production of less highly polymerized acid.

Ragan: Dr. Bauer, at the ACTH Conference in Chicago, reported that he found a good clot, which means he found highly polymerized hyaluronic acid while the patient was receiving ACTH but when the hormone was stopped the polymerization again decreased.

REFERENCES

1 BOAS, N F · Isolation of hyaluronic acid from the cock's comb *J Biol Chem* **181**, *573 (1949).*
2 BLIX, G, and SNELLMAN, O: On chondroitin sulphuric acid and hyaluronic acid *Ark f. Kemi. Mineral. o Geol* **19A**, No 32 *(1945)*
3. RAPPORT, M M, and LINKER, A Unpublished experiments
4 GERSH, I., and CATCHPOLE, H R.. The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth *Am. J Anat* **85**, *457 (1949).*
5 ALTSHULER, C H, and ANGEVINE, D. M: Histochemical studies on the pathogenesis of fibrinoid *Am J. Path* **25**, *1061 (1949)*
6 SYLVÉN, B Ester sulfuric acid in stroma connective tissue *Acta Radiol* **32**, *11 (1949)*
7 MICHAELIS, L, and GRANICK, S Metachromasy of basic dye stuffs *J Am Chem Soc* **67**, *1212 (1945)*
8 HOLMGREN, H, and WILANDER, O · Beitrag zur Kenntniss der Chemie und Funktion der Ehrlischen Mastzellen *Zeit. f. Mikr anat Forsch* **42**, *242 (1937).*
9 SYLVÉN, B Über das vorkommen von Hoch mole Kularen *Esterschwefelsauren im Granulationsgewebe unde bei der* Epithelregeneration *Acta Chirurgica Scand* **86**, (Supplement 66) *1 (1941)*
10 CAVELTI, P A · Studies on pathogenesis of rheumatic fever, cardiac lesions produced in rats by means of autoantibodies to heart and connective tissue *Arch Path* **44**, *13 (1947).*

ENZYMATICALLY MODIFIED OVALBUMINS

GERTRUDE E. PERLMANN
Department of Protein Chemistry
Rockefeller Institute for Medical Research

SEVERAL YEARS ago, while working in Dr Albert Fischer's Institute in Copenhagen, I became interested in developing methods which would make it feasible to modify a few characteristics of a protein molecule in order to learn something about the structure of these materials. The first attempt was a chemical one and can be compared with the histological staining reactions discussed by Dr. Angevine and Dr. Bennett yesterday. Dr. Heinz Herrmann and I studied the reaction of proteins, i e., ovalbumin, serum albumin, edestin, insulin and a few others, with precipitating reagents such as metaphosphoric acid, trichloroacetic acid, sulfosalicylic and picric acids as well as certain dyes When these substances are added to a protein solution, an insoluble, amorphous precipitate is formed In the case of metaphosphoric acid, the reagent which was most thoroughly studied, the phosphorus content of the precipitate equals the total number of positively charged groups of the protein (1,2,3). If the protein is present in excess, so that only a few positively charged groups combine with metaphosphoric acid, ovalbumin (4), serum albumin and edestin (5) crystallize as metaphosphate complexes. Although it is thus possible to alter an essential property of the protein, namely, the solubility, without destroying its crystallizability, the reaction is nonspecific and involves many different amino acid residues of the molecule.

Some years later, I had the opportunity of collaborating with Dr. Goebel, of the Rockefeller Institute, in the study of another chemical reaction. As discussed yesterday, it is generally assumed by histologists that periodate is a specific reagent for carbohydrates Dr. Goebel, Dr. Olitzky and Dr. Saenz (6), however, had shown that periodate destroyed the biological activities of crystalline ribonuclease and the immune globulin of type III antipneumococcus horse serum. In order to find out with which groups of a protein this reagent reacts, crystalline bovine serum albumin was

subjected to the action of dilute lithium periodate (7). It was found that destruction of the amino acids, cystine, cysteine, tyrosine, and tryptophane occurs. Consequently, it is not surprising that this was accompanied by a change in the absorption spectrum and in the electrophoretic behavior of the protein. Prolonged contact of bovine serum albumin with lithium periodate destroys the ability to elicit antibody formation in experimental animals. Again one had to conclude that this oxidation method, at least, was nonspecific and too drastic.

Enzymatic methods were also considered This approach was first suggested by Linderstrom-Lang (8) and tried experimentally by Svedberg, Tiselius and their collaborators (9,10,11). In the work of the Swedish group, proteins were hydrolyzed with the aid of enzymes such as papain and pepsin. In most cases, the enzyme acted very rapidly and the end product consisted of a mixture of peptides of an average molecular weight of 1,000. It was shown later in this country by Peterman and Pappenheimer (12) and Peterman (13) that if proteolysis is allowed to take place at a pH value somewhat removed from that at which the enzyme activity is a maximum, larger split products are formed. Some of these retain the biological activity of the original protein.

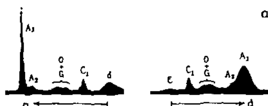
The experiments which I shall discuss now belong to this type of approach. They have been carried out in close collaboration with Professor K. Linderstrom-Lang of the Carlsberg Laboratory and are based on a laboratory accident. To familiarize you with the starting material three electrophoretic patterns are reproduced in Figure 20.

Pattern *a* represents egg white which is a mixture of several proteins (14). From this mixture ovalbumin can be prepared by ammonium sulfate precipitation. It can be readily crystallized but pattern *b* shows that crystalline ovalbumin is electrophoretically inhomogenous and consists of two components, A_1, and A_2, respectively (15). Pattern *c* of Figure 20 illustrates an observation of MacPherson, Moore and Longsworth (16). They found that on aging of a salt-free ovalbumin solution the slower moving component A_2 had increased at the expense of the electrophoretic component A_1.

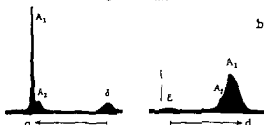
In 1947, Linderstrom-Lang and Ottesen (17), during an attempt to recover ovalbumin from a salt-free, isoelectric solution, observed that the protein when crystallized with ammonium sulfate appeared as beautiful rectangular plates instead of the usual flat needles. Since this solution was six to eight months old, Linderstrom-Lang raised the question of whether or not a relation

Electrophoretic Patterns of Egg White and Ovalbumin in Sodium Phosphate Buffer of pH 6 8 and 0 1μ

Egg White



Ovalbumin



"Aged" Ovalbumin

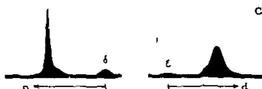


FIGURE 20 Electrophoresis was carried out for 14 400 seconds at 6 4 volts per cm

existed between their findings and the observation of MacPherson, Moore and Longsworth (16) The preparation of the Danish workers was examined electrophoretically at the Rockefeller Institute and the result suggested that the plate albumin was a *new* protein which differed, in its electrophoretic behavior, not only from normal ovalbumin but also from the modification described by MacPherson, Moore and Longsworth.

Linderstrom-Lang and Ottesen immediately considered the question of bacterial contamination as origin of this phenomenon and tested their entire stock of the salt-free ovalbumin solutions that had been on hand for a year or two In the case of three of

Electrophoretic patterns of ovalbumin and plakalbumin in phosphate buffer of pH 6.8, μ 0.1

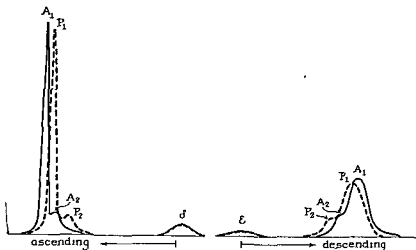


FIGURE 21 * Electrophoresis was carried out for 9,900 seconds at 6 volts per cm

these the toluene which originally had been added as an antiseptic had evaporated and the solutions were contaminated with bacteria. The protein of these three solutions crystallized as plates whereas only needles could be recovered from the noninfected preparations. The Danish workers thus concluded that a bacterial enzyme was active in a reaction leading to a protein which crystallized as plates (*17*). In order to test this hypothesis they added a crude enzyme preparation from *B. subtilis* to freshly prepared ovalbumin and found that within a few hours 95 percent of the protein was transformed into a material that crystallized as plates in the pH range from 5.0 to 5.5. They named this new protein "plakalbumin," "plak" being derived from the Greek for plates. The transformation involved a change in the crystal form, an increase in the solubility and the liberation of one to two percent of the total nitrogen as nonprotein nitrogen.

My work at the Rockefeller Institute consisted, in part, in a study of the electrophoretic behavior of plakalbumin (*18*).

In Figure 21 are shown the tracings of electrophoretic patterns of ovalbumin before and after treatment with the bacterial enzyme. The full and dashed lines represent ovalbumin and plakalbumin, respectively. Under the experimental conditions in which

*Reprinted from *J Am Chem Soc* 71, 1146 (1949)

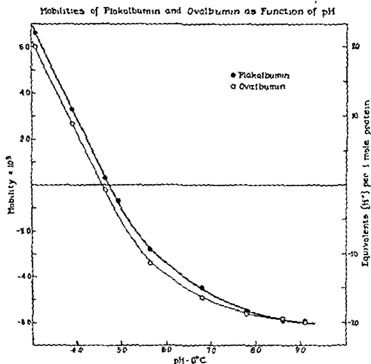


FIGURE 22

potential gradient and time were kept constant, superposition of the patterns permits a direct comparison not only of the relative concentrations of the components, but also of their approximate mobilities. It is thus clear that although the patterns are similar in their appearance, the plakalbumin has a lower mobility, u, than *its parent substance ovalbumin* The actual values are -5.9×10^{-5} and -5.5×10^{-5} for the main components A_1 and P_1, respectively, and -4.8×10^{-5} and -4.3×10^{-5} for the components A_2 and P_2.

Figure 22, a plot of the mobility, u, as ordinate against the pH as abscissa, illustrates the electrophoretic behavior of the predominant components of the two proteins over the pH range from 3.0 to 9.0. From the relative position of these curves it is apparent that *the isoelectric pH values of the two proteins are different*. Thus, ovalbumin in an acetate buffer of 0.1 ionic strength is isoelectric at pH 4.58 and plakalbumin at pH 4.72. Since there is considerable evidence for a constant proportionality

between mobility and the net charge of the protein ion (19,20). the shift in the isoelectric pH which accompanies the A ⟶ P transformation was used to compute the change in the net charge. A value of −2 was found, i.e., two negatively charged groups were lost during the A ⟶ P process.

While this work was in progress, Eeg-Larsen, Linderstrom-Lang, and Ottesen (21) identified the fragments that are lost in the ovalbumin-plakalbumin transformation as two peptides, one of which contains glycine, alanine and valine, the second glutamic and aspartic acid. Thus the change in the net charge is in good agreement with the chemical results of the Danish workers.

Since it was thus established that plakalbumin differed electrophoretically not only from its parent substance, ovalbumin, but also from the protein reported by MacPherson, Moore, and Longsworth (16), further studies were directed towards an investigation of the type of reaction which would lead to the modification observed by these workers. Several possibilities that presented themselves as an explanation were soon eliminated. Thus the increase of the A_2 component at the expense of A_1 was not a spontaneous reaction since *sterile*, salt-free ovalbumin solutions could be stored for one to two years at 3°C. without any change in the electrophoretic pattern. Furthermore, the transformation was not catalyzed by an enzyme originating from the egg white itself. Such an enzyme would have passed a bacteriological filter and thus could have acted on the protein.

I then tested an unsterilized, salt-free ovalbumin solution which had been stored at room temperature for four mouths. Superficially the electrophoretic pattern (22) resembled that obtained by MacPherson, Moore and Longsworth except that the protein crystallized as plates and had a higher solubility than normal ovalbumin. Dr. R. C. Lancefield of this Institute kindly tested this material for bacterial contamination and found rod-shaped, gram positive bacteria to be the containment, probably *B. subtilis*.

Since the contaminnment of this solution may have been a different strain of *B. subtilis* than the one used by the Carlsberg group in their preparation of the plakalbumin enzyme, the possibility could not be excluded that these bacteria contained enzyme systems which catalyze a different type of protein transformation. This point was tested in the following manner: a salt-free ovalbumin solution of pH 4.9 was inoculated with the bacteria and samples were analyzed after certain time intervals. The results are summarized in Table II.

TABLE II

Bacterial Degradation of Ovalbumin

Incubation at 37° C days	Crystal form	Concentrations of electrophoretic components in percent					% non-protein nitrogen	Mg P per q N
		A_1 -5 9	A_2 -4 8	P_1 -5 5	P_2 -4 4	Q -3 6		
0	Needles	83 4	16 6					$8\ 2_8$
14	Plates			84 1	15 9		1 2	$8\ 2_0$
17	"			43 4	41 5	15 1	15 7	$5\ 4_0$
20	"			29 7	52 3	18 0	21 1	$5\ 1_8$
26	"			16 1	57 2	26 7	37 7	$4\ 1_0$
35	"			10 8	56 6	32 6	51 0	$3\ 9_0$

The first column of this table indicates the incubation period at 37°C., the second the crystal form of the isolated protein, the third the electrophoretic results. The electrophoretic mobilities were used for the identification of the different components of the mixtures and are listed as the headings on this column Column 4 and 5 give chemical data It is apparent from this table that no changes occurred during the first 10 days. After 14 days, however, a transformation of A_1 and A_2 into P_1 and P_2 took place. The change of crystal form, the liberation of 1.2 percent of the total nitrogen as nonprotein nitrogen and no change in the phosphorus content indicated that this process was identical with the A ⟶ P transformation and the protein was pure plakalbumin (18, 23).

Further exposure of the protein to the bacteria resulted in a) a decrease of the P_1 component, b) an increase of P_2, c) the apperance of a slowly moving component, Q, in the pattern, d) decrease in total protein and, most significantly, e) a decrease in the phosphorus content of the protein. This suggested that the degradation of ovalbumin with our strain of *B. subtilis* involved both proteolysis and dephosphorylation (25).

It had been shown by Sorensen and his collaborators in 1926 that ovalbumin is a phosphorus-containing protein that can be separated by stratified electrodialysis into a phosphorus-poor and a phosphorus-rich fraction (24) In view of these facts and the electrophoretic inhomogeneity later substantiated by Longsworth (15), Linderstrom-Lang has pointed out that the phosphorus content of this protein does not correspond to an integral number

of phosphorus atoms per mole of protein if a molecular weight of 44,000 is assumed. Professor Linderstrom-Lang furthermore suggested to me in 1947 that the fast moving electrophoretic component, A_1, should contain two atoms of phosphorus per mole of protein, the slower one, A_2, only one atom of phosphorus (23). Thus, an ovalbumin preparation with an electrophoretic composition, as given in line 1 of Table II, has a phosphorus content of:

$$2 \times 0.834 + 0.166 = 1.8_3 \text{ atoms per mole.}$$

The analytically determined value of 8.29 mg phosphorus per gm. of protein nitrogen corresponds to:

$$(8.29 \times 6.94)/31 = 1.8_5 \text{ atoms per mole.}$$

If now the assumption is made that P_1 of plakalbumin also contains two atoms of phosphorus, P_2 one atom and Q no phosphorus, a comparison of the values computed for this element with the aid of the electrophoretic analysis and the chemically determined value is given in Table III:

TABLE III

Comparison of the Phosphorus Content of Enzymatically Altered Albumin with Electrophoretic Analysis

Incubation at 37° C	Atoms phosphorus per 44,000 g protein	
days	found	calculated from pattern area
0	1.8_5	1.8_3
14	1.8_3	1.8_4
17	1.2_0	1.2_8
20	1.1_4	1.1_2
26	0.9_1	0.8_9
35	0.8_7	0.7_8

A_1, P_1 contain 2 atoms P per mole protein
A_2, P_2 " 1 atom " " " "

The agreement is excellent.

This type of experiment did not, however, reveal the reaction leading to the ovalbumin modification of MacPherson, Moore and Longsworth (16). Consequently, the hypothesis emerged that a dephosphorylation process was the origin of the $A_1 \longrightarrow A_2$ transformation described by these authors. This was tested as follows:

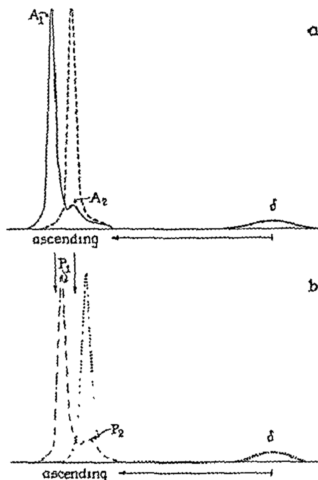


FIGURE 23* Electrophoretic patterns of ovalbumin and plakalbumin before and after dephosphorylation in sodium phospate buffer of pH 6 8 and 0 1 μ

LEGEND

a) — ovalbumin, ---- dephosphorylated albumin
b) ····· plakalbumin, dephosphorylated plakalbumin after electrophoresis for 12,600 seconds at 6 4 volts per cm

*Reprinted from *Nature* 166, 870 (1950)

Highly purified prostate phosphatase, kindly furnished by Dr. Gerhard Schmidt, of the Boston Dispensary, was added to a salt-free ovalbumin solution of pH 5.4. The electrophoretic component, A_1, was converted, with a loss of phosphorus into a component having the mobility of A_2. This protein crystallizes as needles and contains one atom of phosphorus per mole. Using plakalbumin as the substrate, P_1 is transformed into a protein with the mobility of P_2 that crystallizes as plates. In Figure 23a are superimposed the tracings of the electrophoretic patterns of ovalbumin before and after treatment with prostate phosphatase, whereas Figure 23b shows the patterns of plakalbumin before and after dephosphorylation. The arrows in Figure 23b indicate the boundary positions that A_1 and A_2 would have if these proteins were present.

If A_2, prepared by dephosphorylation of ovalbumin, is now treated with the proteolytic enzyme from *B. subtilis*, about two percent nonprotein nitrogen is liberated. The resulting protein crystallizes as plates, contains one phosphorus atom per mole and has the same mobility as P_2.

These results make it appear likely that the ovalbumin modification of MacPherson, Moore and Longsworth (16) represented a partially dephosphorylated ovalbumin, whereas the modification which I reported in 1948 (22) and which crystallized as plates was dephosphorylated plakalbumin Preliminary experiments in which a salt-free ovalbumin solution of pH 4.9 was inoculated with *B. subtilis*, but to which toluene was added, indicate that under such experimental conditions the proteolysis is depressed and the dephosphorylation mechanism enhanced (26).

The dephosphorylation with prostate phosphatase is accompanied by the formation of about 0.2 to 0.4 percent of nonprotein nitrogen. This may be due to the action of traces of proteolytic enzymes present in the phosphatase preparations. However, at the present time it cannot be excluded that an amino acid is liberated, i.e., that part of the phosphate in ovalbumin represents a nitrogen-phosphorus linkage.

If preparations of A_2 and P_2, respectively, are treated with an intestinal phosphatase, further dephosphorylation occurs which is accompanied by the appearance of a slower moving component A_3 and P_3, respectively, in the electrophoretic pattern of the proteins. It has not been possible yet to prepare pure A_3 or P_3 but chemical analyses of several mixtures indicate clearly that A_3 does not contain phosphorus. From these results the scheme presented in Figure 24 emerges.

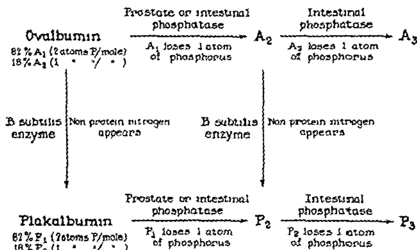


FIGURE 24 Schematic representation of reactions leading to modified ovalbumins

As can be seen, with the selection of only three enzymes, five different ovalbumin modifications can be obtained They all are crystallizable, differing in their electrophoretic properties and in some of their chemical characteristics. Moreover, it can be concluded that a difference in the chemical nature of the two phosphorus containing groups of ovalbumin exists (27).

In conclusion it should be pointed out that such enzymatic methods which induce minute changes in a protein molecule may also prove to be of value in altering the solubility of so-called structural proteins, like collagen and elastin, and thus make it possible to investigate their chemical properties

Jones Did you use the second preparation, the one with one phosphorus, with subtilis enzyme?

Perlmann. Yes, I have done that too

Fremont-Smith. Does that change it?

Perlmann Yes, it changes. I will come to that later.

Mirsky. Dr. Perlmann, I have a block right now on the names of the workers in the field. Were there not reports in which a dephosphorylating proteolytic enzyme has been reported with reference to sea urchin eggs, or frogs' eggs?

Perlmann Reports in the literature state that phosphatases prepared from mammalian tissue do not dephosphorylate phosphoproteins. Phosphoprotein phosphatases were demonstrated in

citrus fruit by Axelrod and in frogs' eggs by Harris. The enzyme preparation described by Harris and the more recent one of Feinstein contain proteolytic enzymes as impurities. In our work we wanted to be able to rule out *proteolytic activity and therefore* used Dr. Schmidt's preparations.

Mirsky: When you say "phosphatase" have you any specific ones in mind?

Perlmann: Both the prostate enzyme and the intestinal phosphatase are rather unspecific and hydrolyze a great number of monophosphate esters. Schmidt has also shown that the intestinal enzyme dephosphorylates disphosphate esters and pyrophosphate.

Dempsey: What pH was used with the phosphatase preparations, both prostatic and intestinal?

Perlmann: The prostate phosphatase has an activity optimum at pH 5.4. This is very fortunate in our case since this is the pH range in which ovalbumin is most stable. The activity optimum of the intestinal enzyme in case of low molecular weight substrates is at pH 9.0. This enzyme, however, dephosphorylates ovalbumin, both at pH 9.0 and at pH 5.4.

Meyer: Do you have any information as to whether the phosphate is bound on serine or another hydroxyamino acid?

Perlmann: We do not have any information yet whether or not the phosphate is bound to a hydroxyamino acid.

Meyer: Does the enzyme not act on casein?

Perlmann: According to Dr. Gerhard Schmidt these enzymes do not dephosphorylate casein; casein contains serinephosphate. However, we certainly do not want to rule out the occurrence of serinephosphate in ovalbumin. Serinephosphate may be responsible for part of the phosphorus content.

Mirsky: The enzymes that you have tested do not act on casein?

Perlmann: No.

Jones: What does the enzyme do to the original preparation?

Perlmann: If intestinal phosphatase is added to ovalbumin, dephosphorylation occurs very rapidly until 46 percent of the total phosphorus is set free. At this point A_1 has been transformed to A_2. Dephosphorylation continues, but at a much slower rate. In the plakalbumin formation, the phosphorus content of the protein remains unchanged. During the $A \longrightarrow P$ transformation, aspartic and glutamic acid are liberated. The change of the electrophoretic mobility is adequately explained by the loss of these two amino acids.

Ragan: A_2 has one phosphate?

Perlmann: Yes.

Angevine: At what temperature are these carried out?

Perlmann: The reactions are carried out at 37°C. However, both the proteolytic reaction with the enzyme from *B. subtilis* as well as the dephosphorylation will also proceed at lower temperature, i.e., at icebox temperature.

Gyorgy: It contains two enzymes, proteolytic and phosphatase?

Perlmann: In the degradation of ovalbumin with *B subtilis*, at least three different processes could be demonstrated: a) a specific proteolytic enzyme which causes the A ⟶ P transformation, b) a dephosphorylation, c) a proteolytic process which degrades the protein molecules to small peptides.

György: I understand that you always use pH 5.3?

Perlmann: We chose pH 5.3 mainly to avoid protein denaturation.

Fremont-Smith: I cannot help saying that this confirms some of the remarks I made at the beginning to the effect that in addition to logic there are romantic and accidental aspects to science. Also it reaffirms the statement—I have forgotten who made it—that chance favors the prepared mind. I think this has been really one of the most thrilling and exciting presentations because we begin to deal with the kind of thing in the sense that Szent-Gyorgyi was talking about yesterday, reaching a level where you can modify piece by piece the protein molecule. Isn't that what we are seeing in this controlled way?

Angevine: It is also interesting in this connection that phosphatase is the only enzyme that has been demonstrated histologically in fibroblasts.

Meyer: This may be a pure accident of methodology, that a histochemical method was developed for this enzyme.

Perlmann: We consider these protein transformations as more gentle reactions than the chemically induced reactions usually applied in protein chemistry.

Fremont-Smith: You mean gentle with respect to certain groups?

Mirsky. Dr Perlmann, have you any ideas as to its character? I suppose that if you had, you would have told us about the proteolytic enzyme involved in conversion. Suppose you took a tryptic reaction and stopped it rapidly?

Perlmann: Trypsin added to ovalbumin does not catalyze the A ⟶ P transformation. Trypsin attacks only denatured ovalbumin, and the denatured protein is then degraded to peptides.

Mirsky. Is this a question of stopping it or a question of specificity?

Perlmann: The proteolytic enzyme from *B. subtilis* which causes the A ⟶ P transformation is a specific enzyme.

Meyer: What is the reason to assume it is a proteolytic enzyme? This assumes too much, it appears to me. It could also be an esterase, for example. Are the linkages in protein all peptide bonds? The second question is: How can we picture that one can split off a considerable number of amino acids without changing the specificity of the protein molecule? Does it mean that these six groups are not in the main or repeating unit?

Perlmann: I cannot answer Dr. Meyer's questions. It has been impossible to demonstrate end groups of peptide chains in the ovalbumin molecule. One thus could conceive that ovalbumin contains several peptide chains, but that these peptide chains are held together by small peptides of a size similar to those liberated during the A ⟶ P transformation. Thus far, no end group determinations have been carried out on plakalbumin and on the dephosphorylated proteins. The fact that ovalbumin and plakalbumin cannot be distinguished immunologically only indicates that during the A ⟶ P transformation the centers of the molecule responsible for the antigenicity are not attacked

MacLeod. Don't you think with respect to immunological reactions that you should construct quantitative antigen-antibody curves to see if the shape is different? This might indicate differences which would not appear by absorption.

Perlmann: Quantitative precipitin reactions certainly should be done; however, I feel that our antigens should be purer than the proteins which we have available at the moment.

Ragan: Dr. MacLeod, has it frequently been found that you can change the electrophoretic properties of a protein without changing its immunological properties?

MacLeod: The only previous observation which I know about is the A_1 and A_2, which I think some time ago were shown to be immunologically similar. Is that true, Dr. Perlmann?

Perlmann: Dr. Landsteiner did not observe immunological differences between the electrophoretically separated A_1 and A_2 components of ovalbumin. Of course, in the case of bovine serum albumin which was treated with lithium periodate, we observed a change in the electrophoretic behavior as well as in immunological characteristics.

Meyer: I believe Maurer and Heidelberger just reported at the Federation Meeting that you can partially deaminate crystalline egg albumin without influencing the immunological properties If you remove a phosphate group, you must get a change in electrophoretic properties

Perlmann: It indicates that the phosphate groups of ovalbumin have a greater influence on the electrophoretic mobility than a free amino group.

Fremont-Smith: We have relativity in antigenicity, do we not? Also we have the fact that antigenicity—am I right—now becomes attached to certain areas of a protein molecule, but is not determined by the specificity of the protein, you can change the protein so it is a different protein, provided you don't dislocate a certain part of the molecule, and maintain your antigenicity? Is that right?

Perlmann: Yes.

Jones: It is gentle.

Dempsey: May I ask a question about technique? What kind of methods does one use to determine the end groups of protein?

Perlmann: I am referring to a method developed by Sanger in England and published in the *Biochemical Journal*

Dempsey: So they are essentially destructive methods with the identification of the products?

Perlmann: Degradation methods

Mirsky: Sanger's insulin.

Perlmann: Most of Sanger's work is on insulin However, he also tried his end group determination on ovalbumin

Holbrook: I thought in further discussion of the protein chemistry of connective tissue that what I have to say might interfere with Dr. Ragan's topic but he assures me that that is not so

We became interested a number of years ago in amino acid metabolism of connective tissue disease and selected rheumatoid arthritis as a likely one to study. It was selected for many reasons, too numerous to mention, as a possible indication of a disorder in intrinsic metabolism that might be related to these diseases of connective tissue. Figure 25 illustrates some of our preliminary observations on the relationship of histidine urinary excretion to rheumatoid arthritis. It will be noted that patients with rheumatoid arthritis have a definitely lower 24-hour excretion than normals. We had found that patients with rheumatoid arthritis in remission from pregnancy also had extremely high levels even as compared to normal pregnancies. One patient in jaundice remission had also shown a markedly elevated level with histidine excretion. Patients with active rheumatoid arthritis were hospitalized on metabolic control, careful control levels of histidine excretion were determined, and then cortisone or ACTH administered, with histidine excretion followed through the course of the remission. As you will note, there were striking increases in

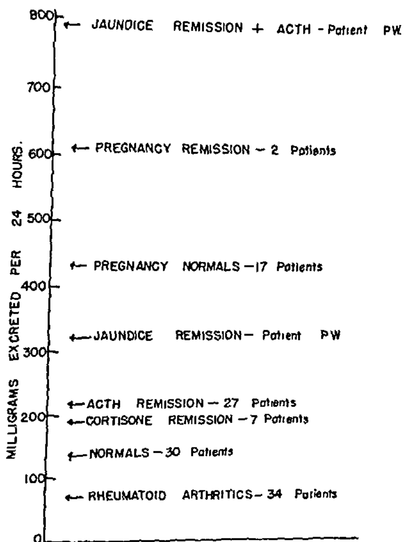

URINARY HISTIDINE EXCRETION
MILLIGRAMS EXCRETED PER 24 HOURS.
800
700
600
500
400
300
200
100
0
← JAUNDICE REMISSION + ACTH - Patient PW
← PREGNANCY REMISSION – 2 Patients
← PREGNANCY NORMALS –17 Patients
← JAUNDICE REMISSION – Patient PW
← ACTH REMISSION – 27 Patients
← CORTISONE REMISSION – 7 Patients
← NORMALS – 30 Patients
← RHEUMATOID ARTHRITICS – 34 Patients

FIGURE 25

urinary histidine excretion when remission of the disease was produced either with cortisone or ACTH. Sixty patients with rheumatoid arthritis have been studied in the hospital under metabolic control with exactly similar findings to the partial results charted in Figure 25. The number of pregnancy remissions studied is now greater but the figures closely parallel those shown in Figure 25. There have been changes in some of the other amino acids; we began by studying 17 but have more recently limited our studies to 8. Histidine is used because the magnitude of the curve is greater than with the other amino acids. Interesting findings were also observed with lysine, arginine, threonine and tyrosine. These, however, have not shown the uniformity in association with remission by cortisone, and by ACTH, in jaundice and in pregnancy that histidine has. So far as we know, these studies on amino acids in rheumatoid arthritis are entirely new.

We are not at all sure whether these histidine changes are related in any way to the remission factor or not. It remains that they do occur with remission and do not occur without remission.

Mirsky: I presume that this data of excretion per 24 hours is related to constant intake?

Holbrook: Yes, metabolic control, all of this. The striking thing that you see there is that whenever a patient undergoes a remission of his disease there is a striking increase in histidine excretion.

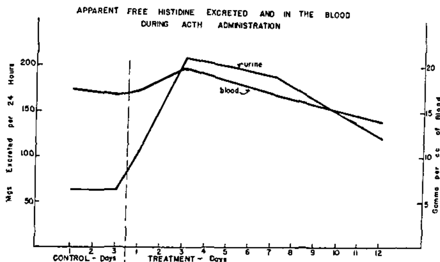
Fremont-Smith: The increase carries above the normal in every case?

Holbrook: Yes.

Ragan: Does that apply to remissions other than those associated with jaundice and pregnancy?

Holbrook: Yes, it applies to remissions achieved with cortisone and ACTH. Gold remissions are more difficult to study. Adequate metabolic controls on patients treated with gold are almost impossible to obtain. Patients who had had remissions on gold or who have obtained spontaneous remissions return to the normal level or exceed it. We have no metabolic controls on gold remissions.

Figure 26 illustrates the type and shape of the curve and something of its magnitude. The histidine excretion curve with ACTH remission is of considerable magnitude and reasonably constant. We have tried a large number of substances following the control period in an attempt to produce this curve—antihistaminics, testosterone, artisone, ATP, adrenaline, thyroid, ascorbic acid, and various combinations. To date we have been unable to



FIGURF 26

produce this curve with any of these substances.

Jones· What dosage?

Holbrook· Testosterone in 100-300 mg. daily. Is there anything else you want to know on dosage?

Jones· Of ascorbic acid.

Holbrook: Ascorbic acid, up to 10 gm., both intravenously and orally, all variations of dose. The curves have been flat in every instance—there has been nothing more than a plus or minus 10 percent. We have also tried amino acid feedings. For a long time we have been laboring under the impression that these curves must be related to diet and to intake, but on treatment day we have started 50 to 100 gm. of hydrolysated protein in addition to the basal diet and never been able to produce anything like this type of curve. There has been a 10 percent increase, perhaps up to 15 percent. Fifteen percent I believe was the highest increase we had on amino acid feeding.

Fremont-Smith. Genuine remission?

Holbrook: No, that is the next point to bring up. In none of

these instances has there been anything approaching a clinical remission. In one instance a remission occurred without this curve. That was a woman who was heavily pretreated with thyroid and ascorbic acid, and throughout the treatment period was given thyroid, ascorbic acid, and ACTH. We later determined that ascorbic acid has a tendency to depress the curve. This may be the explanation for the single exception of a remission

thing that I can say is that in our experience so far it has never occurred except with remission, and that it has failed to occur only once with remission in the case which I told you about.

Mirsky: Does it precede or follow remission?

Holbrook: It happens about the third day. It is simultaneous with remission.

Mirsky: In other words, the clinician records the clinical findings every day, and on the third day he sees that there is a change, namely, the patient is improving.

Holbrook: That is right

Mirsky: Simultaneously, if you give both you find it on the third day?

Holbrook: There may be a day's variation

Mirsky: Which way did the variations go?

Holbrook I cannot answer that I am not sure

Ragan: Does the rise take longer with cortisone than with ACTH?

Holbrook: It is slower. It reaches its peak about the sixth or seventh day.

Meyer: Is it correlated to the sedimentation rate?

Mote: May I interrupt? I recall that Bram Rose in his sensitivity studies observed a marked excretion of histidine during ACTH administration in cases that went into remission (28).

Holbrook: He did some on asthma. That is the only thing I know of that has been reported. The question is: Is this a part of the remission factor? I don't know except that we have been able to produce remission with our small doses in certain cases of bona fide rheumatoid arthritis, without producing any of the other measurable effects that we know how to measure. For instance, we have been able to produce remission in two patients with totally negative Thorne's tests repeated on as little as 30 mg. of ACTH a day Two of those patients receiving 10 mg. a day achieved 100 percent remission with a steadily rising eosinophil

count. Several patients have achieved 100 percent remission without the slightest effect upon the sedimentation rate. Others have had dramatic drops in sedimentation rate with less than the average amount of clinical remission. There is no measurable change in blood pressure, weight, blood sugar levels, sugar tolerance tests, urine sugars and hypertension. It is conceivable that this is only a more sensitive physiological effect of some unknown substance, and has no relationship at all to the remission factor. On the other hand, with such little data as we have in pregnancy and jaundice with gold (though I would not like to report that) and with ACTH and cortisone, it suggests at least the possibility that somewhere in the basic mechanism the amino acids play some role, particularly these four that I have mentioned, in the mechanism of remission. I think that is all I have to say.

Fremont-Smith: Histidine—

Holbrook: Histidine, lysine, threonine, and tyrosine. Some of the others show small variations, plus or minus. The histidine has been by all odds the most constant curve, the most characteristic curve, and of the greatest magnitude. That is the reason I used it.

Mote: Could the histidine excretion be some reflection of histamine mechanism?

Holbrook: We have considered it.

Mote: In the light of Bram Rose's work?

Holbrook: If one wants to make the simple assumption that rheumatoid arthritis is nothing but chronic histamine poisoning, then the simple relationship of histidine to histamine in decarboxylation would be a wonderful explanation. It is so simple that it cannot possibly be true.

Meyer: Why should you then have the others?

Mirsky: That's the whole point.

Holbrook: I don't know. The others do not show anything like these percentages. These percentages that we see in histidine run 200 or 300 percent. It is not a matter of plotting a statistical curve. It is very real and it is of a large magnitude.

Mote: The asthamtics run up at least that high and higher. I am just interjecting this as food for thought.

Holbrook: We don't know anything about this curve in other diseases. We have done a few but have not enough on which to make any report at all.

Travell: What other diseases?

Holbrook: We have played with asthma, we have played with Sjogren's syndrome, we have played with infectious hepatitis, we

have played with various skin reactions, we have played with gold reactions—and interestingly enough ACTH abolishes the gold toxicity immediately—with curves that look something like this, but we have not enough of any one of the other diseases to offer an opinion as to whether or not these curves are the same.

Bennett: Has it been tried on patients with disseminated lupus or with rheumatic fever?

Holbrook: One patient with lupus was studied with a curve very much like this, but still it is not enough to say that this is the curve.

Fremont-Smith: Any remission?

Holbrook: A temporary remission.

Meyer: How many milligrams of histidine or mole excretion per day does that represent?

Holbrook: That is the 24-hour

Meyer: The maximal in the average case?

Holbrook: That is each 24-hour urine collected, and the total amount of histidine.

Meyer: That is roughly—

Gyorgy: One hundred and thirty milligrams is the normal.

Holbrook: Rheumatoids run about 68 mg , normals run around 100 mg , pregnancies run up to 600 mg Those figures are exact figures but are not on there because—

Jones: I did not get what you said. I think you covered it, but you talked about the one exception.

Holbrook: One woman experienced a remission who had been pretreated with thyroid.

Jones: And ascorbic acid. Have you any data on naturally occurring remissions without any therapy?

Holbrook: Do you know how hard that would be to get in hospitals?

Jones: It is important.

Holbrook: I have done estimations on them

Jones: I still think that is important.

Holbrook: We have done estimations on them but there has been no continuity of study and no metabolic control. It is possible now, since we believe that intake is not so important in effecting this curve, that we may be able to get some further information on spontaneous remissions

Jones: The reason I asked this was that the whole enzyme system may be altered by these substances Aside from the effect on decarboxylation of histidine, we just heard about, we have the report of Hamburger who reported that glucuronidase is excreted

in increased amounts early by those rheumatoids who show an effect from ACTH and cortisone. I don't know what the meaning of this is.

Holbrook: This story is simply presented as new information to stimulate thinking and further work with regard to the amino acids in the problem of connective tissues. No conclusions can as yet be drawn.

Mote: I still think you have an intriguing point there, that here you have some patients you put on tremendous doses of hydrolysate, with plenty of amino acids, yet only specific situations in which you get this increased excretion. Furthermore, the other thing that still intrigues me is that the asthmatics and hypersensitivity patients do the same thing in an even more exaggerated form.

Mirsky. In what respect? Low to begin with?

Mote. I don't recall the details of how low, but they went up to 1,000 mg. and more a day excretion.

Mirsky: After administration of cortisone.

Meyer: Some rather large changes in some proteins must take place, for example, in the protein fractions responsible for the sedimentation rate. If these proteins, which apparently are poured out into the circulation in the arthritic patient, no longer appear, are they broken down or are they no longer produced? In allergies, in asthmatics for example, do the eosinophils break down?

Holbrook: They don't always. You may have a remission with a rising eosinophil count.

Mote: Where does that all fit into histamine?

Holbrook: Dr. Meyer is quite familiar with this. He spent a couple of weeks with us and saw all of this work. I think I may say one more word, and then I will stop. This occurs whether the patient is in negative or positive nitrogen balance. It occurs whether you give huge doses of testosterone and high amounts of amino acid feeding, or whether you institute a starvation regimen. It went on constantly negative. As far as we can tell the curves don't vary appreciably from starvation or positive nitrogen balance if remission occurs.

Gyorgy: I would like to add a few remarks. First of all, we may be mistaken if we relate increased excretion of amino acids to disturbed protein metabolism. It is much more probable that the kidney as excretory organ is involved in the finding observed.

Holbrook: Very probably.

Gyorgy: Perhaps the renal threshold is lowered. The blood level

is only very slightly increased, about 20 percent, if I interpreted your chart correctly.

Holbrook: But statistically this change is significant.

Gyorgy: I am, however, not quite convinced that the method used is sensitive enough with regard to a 20 percent increase.

Holbrook. That is right.

Gyorgy: In addition to the kidney, perhaps the liver should also be taken into consideration in explaining Dr. Holbrook's findings.

Holbrook: We have done one study on the kidneys. May I tell you about that? We have done one patient with combined rheumatoid arthritis and rather severe chronic nephritis, running a blood urea nitrogen of around 40 constantly, fairly in equilibrium, neither getting worse nor better, and in that instance the P.S.P was markedly decreased, and the P.S.P decreased further instead of increasing following the ACTH administration However, the amino acid curve increased just the same when he got his remission That is about all I know about the relationship of the kidney to this problem

Gyorgy. I don't mean that you could correlate it with the usual pathologic conditions of the kidneys, such as nephritis, nephrosclerosis, etc. I was thinking of the work of Dent (29) and of ourselves on cystinuria (30). Here amino aciduria limited to cystine, lysine and arginine occurs, probably due to a functional tubular disturbance. The intermediary protein metabolism as such may not be involved in this process at all.

Mirsky: If this is due to a kidney change, the reabsorptive capacity does not appear to be involved.

Holbrook: There is the renal possibility.

Mirsky: If it were, it would not explain why it does not occur in situations other than those associated with remission.

Gyorgy: Why not? That is what Dr. Holbrook found That is a very important finding. I am not questioning the importance of it; I am questioning only the explanation.

Holbrook. That is right.

Mirsky: I am too, at the moment. If this is a question of the Tm of the kidney, or a question of some specific synthesis, I cannot understand why it should not occur in an organism when given an excess, to a nonrheumatoid individual

Gyorgy George Thorn has very clearly demonstrated the importance of adrenal cortex to renal function.

Meyer The total amount probably is very small

Gyorgy: I have the feeling, if I were to put my penny on a bet, it is a renal disturbance.

Meyer: Why don't you have an elevated blood concentration?

Gyorgy: You don't have with the renal. You may have a glycosuria and have low blood sugar values.

Mirsky: I am talking about tubular function.

György: Yes.

Mirsky: The reason I am thinking in that direction is that we know, for instance, that cortisone and ACTH affect the reabsorptive capacity of the kidney very early. Recently we obtained some evidence that the absorption of glucose after ingestion is influenced by cortisone and ACTH. But what disturbs me is I would expect the same phenomenon to occur in the individual who does not have rheumatoid arthritis and also in the one with rheumatoid arthritis who does not develop a remission.

Mote. John Browne and Bram Rose did a number on some normals and did note as I recall, some shift (31). The thing that struck them was the magnitude of the shift in the asthmatic particularly. They felt it was beyond the expected renal threshold.

Gyorgy: Both kidney and liver, I would say. They are linked so closely together.

Mirsky. The important thing to understand, is whether we are dealing with a catabolic phenomenon, i.e., increased proteolysis, or are we dealing with cessation of synthesis, or is it a combination of both?

Ragan. If you can answer that you have answered a very fundamental question.

Mirsky: I like to ask fundamental questions.

Holbrook. That is the way we get started on this.

Dempsey: Is histidine excretion increased in pregnant animals? Because if it is, one has an experimental situation in which the liver can be removed, the kidney altered, and so on. One could study the physiological mechanism whereby this elevation of histidine is brought about. One might find out whether it is caused by an increased protein breakdown under the influence of adrenal hormones, or whether there is a decarboxylation and so forth.

Fremont-Smith: Could you do a histidine clearance before and after ACTH and see whether you could demonstrate a change in renal threshold?

Holbrook: We have some tolerance studies set up that we are playing with but we have nothing to report yet.

Ragan: I might say the pregnancy remission is probably due to ACTH, because Dr. Jailer at Columbia has shown recently that the placenta is a very potent source of ACTH-like material.

Dempsey. Along that line Venning has shown that the adrenal corticoids are raised during pregnancy (32). I remember asking last night whether ACTH in a normal individual gave this elevation.

Holbrook· About 25 percent roughly.

Dempsey: So I thought it would probably be easier to get at it experimentally in a pregnant animal than to try to alter it in the human.

Ragan: With large doses of ACTH, do you approximate the pregnancy level?

Holbrook. We have attempted to determine whether there is a direct quantitative relationship in the histidine curve to the size of the dose. There appears to be a somewhat rough quantitative relationship. We have worked, however, very little with high doses so that we have insufficient data to be certain on this point. It does, however, appear that in those where there was an abrupt and dramatic remission of the disease, the histidine curve appeared to be higher. Much more work will be needed to determine the relationship of the quantitative response to larger doses of ACTH as compared to the histidine response on dramatic remission.

Harrower: You had a group of normal patients who had the same disease, what constitutes normality? How do they differ from the control group?

Holbrook: It was simply that so far as we knew the people had no complaints, and were people who had been examined, chest x-[illegible] to do the study, when we found they were normal, but they may not have been normal. Dr. Harrower, I would readily admit that but they didn't have any known disease.

Harrower No one group characteristic?

Holbrook: I think it is fair to say the number of normals was small. We will have to do a good many more normals before we can say without any question that their curves are definitely lower than this.

Mirsky: In view of our present knowledge we could assume that with increased corticoid secretion there would be an increase in protein catabolism and increased proteolysis, a release of amino

acids for gluconeogenesis and incidentally for excretion. So, it is not farfetched to expect this type of response to the administration of ACTH or cortisone. But I would expect it to occur in patients who have no disease. These observations are exciting because they suggest a specific biochemical derangement in a patient with rheumatoid arthritis.

Holbrook: I am not sure it is specific. I said that very plainly.

Mirsky: By "specific" I don't mean a different disease. Hitherto, there has been no evidence of adrenal insufficiency.

Gyorgy: Marrion recently published a paper in which he states that pregnanediol excretion is increased in rheumatoid arthritis which may again lead to the liver as the disturbed organ (33). You probably have seen that.

Ragan: He did not specify whether the patients were on salicylates. You will find an increase in glucuronic acid excretion if salicylates have been administered.

Meyer: The difference between the normal and the rheumatoid from the figures I jotted down is an average of 130 mg. per 24 hours to approximately 70 mg. in the latter.

Holbrook: Right.

Meyer Do you know whether chronic starvation has any effect?

Holbrook. I do not believe that chronic starvation will have any direct influence on this curve as some of the patients were markedly malnourished.

Mirsky: Irrespective—

Holbrook: We have done no starvation studies.

Mirsky: I am referring to rheumatoid arthritis responding differently to ACTH and showing some biochemical disturbance and, in the collagen diseases, responding differently.

Holbrook: It is not fair to say that yet. I would like to make that point. We have not investigated enough other diseases to know whether that happens in other diseases. It is not fair to say that yet.

Mirsky. As related to controls.

Mote. You still have Bram Rose's reports on hypersensitivity diseases.

Mirsky. Another related disease.

Holbrook: Maybe a whole group of diseases is involved.

Gyorgy: Has anybody done any chromatography?

Holbrook: We tried it at the University and felt that our quantitative relationships were not good.

Gyorgy: Not for quantitative data?

Holbrook: Quantitatively. We did only a few studies but were not impressed with their accuracy.

Gyorgy. Are there unusual amino acids which were excreted as peptides?

Holbrook: There was nothing different and not a quantitative relationship.

Ragan: Have you had any rheumatoids to whom you have given a high protein diet? Does that raise their histidine excretion toward normal?

Holbrook: It does not. Neither does feeding them protein hydrolysates at that time raise it.

Ragan· Before treatment?

Holbrook: No, we have tried that.

Mote: What about your couple of arthritics, where you had to go to a high dosage and did not get much in the way of remission?

Holbrook. One patient on a high dosage secured measurable remission. She was, however, a psychological problem and denied any improvement whatever.

Travell· Did you get a curve in that patient?

Holbrook· Yes, we secured a curve in the presence of objective clinical improvement and in the presence of the patient's loud denial that she had improved. She did, however, develop some toxic manifestations which may have played a role. She developed edema, hypertension, and mental anxiety.

Travell: Did she show a change *in* the histidine excretion, or was that not studied?

Holbrook: Yes, the swelling disappeared and she had all of the things you can measure as remission physically. I cannot give you that figure.

Mirsky: High, low, normal?

Holbrook· Medium. We have used lately some low sodium diets on our longterm maintenance cases which I have not discussed here because it has nothing to do with the chemistry we are talking about but all these metabolic studies did not have either a low or high sodium intake I cannot give you that.

Fremont-Smith: The patient you had to push up to 100 mg. daily before you got the remission?

Holbrook: Some patients can be pushed up on their dosage and some cannot. It was one of our early cases and I believe we gave her excessive amounts of ACTH.

Fremont-Smith. I thought you mentioned another patient

Holbrook: On cortisone.

Fremont-Smith: In that one was there a delay in the histidine curve corresponding to remission?

Holbrook: That is correct.

Fremont-Smith: So it was only when you reached the high dosage, that you obtained the remission and the curve?

Holbrook: I did not get it until the eleventh day.

Fremont-Smith: I think that is a very significant correlation

Mirsky: The important thing is that one could utilize this technique to find therapeutically effective dose and not go beyond

Holbrook: When you have thousands of test tubes it is not a very practical way to do it.

Mirsky: Histidine may be measured by other methods. That becomes purely a technical problem. If the histidine response becomes a criterion of therapeutic effectiveness rather than a criterion of response to a specific drug then it will be most useful.

Fremont-Smith: The maximum needed for therapy.

Holbrook: I am much more interested in where the amino acids come from. Can anybody help us in trying to find that out?

György: Dent has an ingenious hypothesis regarding the simultaneous presence of cystine, lysine and arginine in the urine of patients with cystinuria. He assumes that amino acids are phosphorylated before or during tubular reabsorption. There is a distinct steric similarity between cystine, arginine and lysine, all three being diamino acids. The molecular distance between the amino groups, expressed in Angstrom units, must be not too different in these three amino acids. This may explain the selective difficulty in phosphorylation for these compounds. Thus, in final analysis, this specific type of amino aciduria could rightly be considered as of renal origin. Whether similar considerations should apply to the amino aciduria seen after administration of ACTH, cortisone or in other conditions of increased adrenal cortical action remains to be seen.

Mirsky: Dr. Holbrook's data would suggest it is not merely a question of reabsorptive capacity of the renal tubules.

György: We don't know. In cystinuria it is either excretion or reabsorption.

Mirsky. Not intraglomerular, tubular.

György. Tubular.

Mirsky: Specific excretion.

Holbrook: How do you spell the name?

György: Charles E. Dent, London University College of Medicine. I don't think he has ever published his theory. The summary

given by me is based on personal communication.

Mirsky: In most of the increased amino acidurias we are dealing with disturbance in reabsorptive mechanisms, aren't we?

György: We don't know, but very probably, yes

Mirsky. Not glomerular?

György. No.

Ragan: In the Fanconi syndrome the problem is one of reabsorption.

Mirsky: I was thinking chiefly about the reabsorption because when an hydrolysate is given, as by Dr György, essentially a clearance test is being performed.

György: Not necessarily. It may not reach the blood It may be filtered off in the liver.

Ragan. We have talked about increased protein catabolism All we have is Kinsell's work on S-35 methionine incorporated into protein (34). All work using excretion as evidence fails to determine whether increased catabolism or decreased anabolism is the primary effect.

Meyer. Assuming that the sedimentation rate is caused by certain proteins and it drops rather fast—in three days—what happens to these proteins?

The total amount of protein presumably would be small, calculated from the histidine excreted. The difference between 130 to 70 mg., which is 60 mg., and the difference of 200 to 70 mg for the cortisone treated rheumatoids in 24 hours is 130 mg. in 24 hours. Assuming a histidine concentration of 6 percent to 8 percent as an average figure for proteins, then the difference of 60 mg. between normal and arthritic would be about 500 mg. of protein and the difference between the rheumatic and the treated rheumatic would correspond to about 1.2 gm. of protein. That is not much.

Mote: In my estimation much of this effect is occurring at what I call a cellular level, and when you stimulate the adrenal gland in some of these syndromes, it still intrigues me as to whether or not histamine is involved in this whole problem, and if so, where and what the relation of that is to histidine excretion

Holbrook: I have not done histamine levels.

Fremont-Smith. Can you get a labelled histidine?

Meyer: Sure!

Fremont-Smith: If you were to feed it, would it remain labelled? If it were incorporated in protein could you feed labelled histidine to these patients beforehand and get a remission with ACTH and see whether your labelled histidine came out?

Would that tell you that it was broken down?

Meyer: It depends how you labelled it.

Fremont-Smith: If you could label it in the right way.

Meyer: In patients the only way you could label it would be with heavy nitrogen. If you labelled histidine in the imidazole ring, you could see whether it is incorporated into the protein. From isolated protein fractions, you would have to isolate the imidazole and see whether it still contains the label.

Fremont-Smith: You could also in animals use radioactive material.

Gyorgy. C^{14}.

Fremont-Smith: You can get an animal experiment with ACTH and there is the possibility of playing with both of these things

Meyer: In animals you could use C^{14}. I don't know whether you could isolate enough histamine.

Mirsky: Here you are dealing with more than histamine. From the data that are given we are dealing with more than just one amino acid. Consequently the whole problem of proteolysis is involved.

I want to speak about Dr. Ragan's statement about not being able to show whether one is dealing with catabolism or anabolism. Even *in vivo* in experimental animals it is quite possible to distinguish the two. In the eviscerated animal one can demonstrate whether you are dealing with catabolism or anabolism.

Gyorgy. Ingle's work.

Mirsky: Ingle's work is an example of that. All the evidence suggests that cortisone stimulates protein catabolism which would fit in with these observations, except for the question as to why it does not occur in the nonarthritic patient.

Holbrook: It may. That is the point I am making.

Mirsky: The nonarthritic, the so-called healthy patients?

Ragan: An eviscerated animal may be utilizing his own proteins and unless the animal is kept in balance, the problem of increased catabolism or decreased anabolism cannot be solved

Mote. I think another problem that is pertinent here is the phenomenon of blocking toxic agents What is the mechanism of this? How does that fit in the protein catabolism or cellular reaction to toxic agents and what is the mechanism of it? It is very peculiar to me that all of a sudden one may block the toxicity of various chemical agents as far as tissues are concerned. Of course one always has to think about hypersensitivity when you say this, as it is the case with a metal like gold, but then there is the

observation of Allan Wood that cortisone will block the inflammatory reaction that occurs when glycerine is instilled in the eye (35) and Cluxton's observation that the toxic effect of the black widow spider can be blocked by the administration of ACTH (36). One may of course consider that there is a hypersensitivity element in all of these phenomena. One can always answer, too, that there is a hypersensitivity element in it, but by the time you get through talking about toxic agents maybe there isn't anything except hypersensitivity as far as the toxic reaction of a material is concerned.

Mote: Such as gold. One can do away with the toxicity of that metal in more than the usual doses while the patient is on ACTH. There is preliminary information that this may be true of other heavy metals as well.

Meyer: You influence the toxic reaction. I don't know whether the explanation is right. You do the same as you do with BAL You have available cysteine. The level in the organs or the heavy metal and therefore you get a detoxifying action. Whether this explanation is correct I don't know, but it is a possible explanation.

Holbrook: I would be much more inclined to think that it merely blocks the hypersensitivity mechanism.

György: That is right. Have you ever seen it in lead poisoning?

Holbrook: I don't know.

Mote: I don't know. It has not been done to my knowledge

György: No hypersensitivity in lead, is there?

Holbrook: We have had four patients with gold reactions who cleared up immediately on the use of ACTH. They did not suffer of course the exacerbation of their disease that you get if you give them a whole lot of BAL. They get worse if you give BAL.

Fremont-Smith: What is the gold reaction?

Holbrook: Skin reaction is what I am talking about, the skin reaction.

György: Exfoliative dermatitis or its precursor.

Holbrook: I am not talking about blood reactions or mouth reactions. These are skin reactions which I think are hypersensitive in nature, whatever that means. Whether it does anything else or not I don't know, but certainly the skin reaction disappears like magic, and the patient feels extremely well and you can go on giving gold.

Fremont-Smith: Does BAL give a fairly regular exacerbation?

Holbrook: If you give enough of it and the patients are in re-

mission from gold—and they usually are about the time they get the reaction.

Gyorgy: Dr. Holbrook, to come back to your problem, I think the most important would be to use the intravenous tolerance test with histidine That is the most important.

Holbrook: We have that set up. I think that is right. We have not done that yet.

Mirsky. In terms of the clearance?

György: Yes.

Holbrook: We will talk to Dr. Meyer when he comes out to visit us and try to work out something.

Mirsky. Could we not take his data right now? Assuming that these patients have normal kidneys, one can calculate the filtration rates, and from the free histidine in the blood calculate the amount of histidine getting into the tubule and determine whether we are dealing with a tubular reabsorption since we have before us the quantity of histidine in the blood and urine.

Gyorgy: I think you still will have to do it on the clearance basis.

Mirsky: That is the evidence that will prove it. I think we have the data here from which we can make some deductions One has to be cognizant of the cause of the effect on other reabsorptive mechanisms in the kidney by cortisone.

Ragan: I might say that in one case of hypertension treated by Dr. Perera with 200 mg. of cortisone acetate a day, tyrosine appeared in the urine.

Gyorgy. Dog?

Ragan. Man.

Holbrook: Tyrosine?

Ragan: Over a month's period.

Holbrook: This was presented not as the answer but to suggest ways and methods of learning a little bit more about it. That is all.

Mirsky. I think it is important to get a few more nonrheumatoids, so-called control, healthy patients.

Holbrook: We have not done it yet. We hope we now can obtain a little more material, and I think we can get some normals. We want to run some other diseases of connective tissue with problems that are interesting to us. We hope to accumulate enough of them to determine the real significance of the type of curve they have.

Jones: Don't you think it would be important to study some nonconnective tissue diseases?

Holbrook: Undoubtedly.

Mote: Again you get into the definition of connective tissue disease. Take the hypersensitivities; if they live long enough progressive fibrosis of various types may occur. Are these connective tissue diseases or what?

Meyer: You get away with it by calling it noninfective connective tissue disease. Call it mesenchymal.

Jones: In real serum sickness the patient will be convinced all tissues are involved.

Mote: Your connective tissue is simply reflecting the cellular environment of the rest of the tissue perhaps

Mirsky: So far as I know, in diabetes there is no disturbance of the amino acid excretion pattern. I draw attention to the fact because I am wondering whether in the patient with diabetes we may not be dealing with some factors that oppose the action of cortisone. Sprague has shown, for example, when the diabetic becomes acidotic there will be increase in the cortisone excretion

Mote: But you can give ACTH or cortisone to a diabetic and not hurt him.

Mirsky: I know that.

Ragan: You can make him awfully diabetic.

Mote. You can make him awfully hyperglycemic, but in my opinion not diabetic in the usual sense of the word

Ragan: Awfully intolerant of a glucose load.

Mirsky: It just puts a lot of sugar in the urine.

Mote: On the other hand he eats perfectly well and feels perfectly well with that terrifically high blood sugar and he does not get acidotic.

Mirsky: You can do that by giving sugar and not make him worse. I am using the susceptibility to ketosis as a criterion of severity. It is not increased.

Dempsey. I was wondering whether there was any connection between heavy metal poisoning and the paradoxical effect of adrenalectomy on skin and hair growth. After adrenalectomy, most functions are impaired, but hair growth is increased and the O_2 consumption of skin is elevated. Skin and hair suggest SH groups. Isn't it possible that if hair were laid down more rapidly, the pool of SH groups might be decreased and therefore heavy metal poisoning would occur more readily — that is, a given amount of heavy metal would have a greater effect than it would if the pool were normal. Conversely, to give cortisone to stop hair growth might increase the SH pool and repair the deficiency caused by heavy metals.

mission from gold—and they usually are about the time they get the reaction.

György: Dr. Holbrook, to come back to your problem, I think the most important would be to use the intravenous tolerance test with histidine. That is the most important.

Holbrook: We have that set up. I think that is right. We have not done that yet.

Mirsky: In terms of the clearance?

György: Yes.

Holbrook: We will talk to Dr. Meyer when he comes out to visit us and try to work out something.

Mirsky. Could we not take his data right now? Assuming that these patients have normal kidneys, one can calculate the filtration rates, and from the free histidine in the blood calculate the amount of histidine getting into the tubule and determine whether we are dealing with a tubular reabsorption since we have before us the quantity of histidine in the blood and urine.

Gyorgy: I think you still will have to do it on the clearance basis.

Mirsky: That is the evidence that will prove it. I think we have the data here from which we can make some deductions. One has to be cognizant of the cause of the effect on other reabsorptive mechanisms in the kidney by cortisone.

Ragan: I might say that in one case of hypertension treated by Dr. Perera with 200 mg. of cortisone acetate a day, tyrosine appeared in the urine.

György: Dog?

Ragan: Man.

Holbrook: Tyrosine?

Ragan: Over a month's period.

Holbrook: This was presented not as the answer but to suggest ways and methods of learning a little bit more about it. That is all.

Mirsky: I think it is important to get a few more nonrheumatoids, so-called control, healthy patients.

Holbrook: We have not done it yet. We hope we now can obtain a little more material, and I think we can get some normals. We want to run some other diseases of connective tissue with problems that are interesting to us. We hope to accumulate enough of them to determine the real significance of the type of curve they have.

Jones: Don't you think it would be important to study some nonconnective tissue diseases?

Holbrook: Undoubtedly.

Mote: Again you get into the definition of connective tissue disease. Take the hypersensitivities; if they live long enough progressive fibrosis of various types may occur. Are these connective tissue diseases or what?

Meyer: You get away with it by calling it noninfective connective tissue disease. Call it mesenchymal.

Jones: In real serum sickness the patient will be convinced all tissues are involved.

Mote: Your connective tissue is simply reflecting the cellular environment of the rest of the tissue perhaps.

Mirsky: So far as I know, in diabetes there is no disturbance of the amino acid excretion pattern. I draw attention to the fact because I am wondering whether in the patient with diabetes we may not be dealing with some factors that oppose the action of cortisone. Sprague has shown, for example, when the diabetic becomes acidotic there will be increase in the cortisone excretion

Mote: But you can give ACTH or cortisone to a diabetic and not hurt him.

Mirsky: I know that.

Ragan: You can make him awfully diabetic

Mote: You can make him awfully hyperglycemic, but in my opinion not diabetic in the usual sense of the word

Ragan: Awfully intolerant of a glucose load.

Mirsky. It just puts a lot of sugar in the urine

Mote: On the other hand he eats perfectly well and feels perfectly well with that terrifically high blood sugar and he does not get acidotic.

Mirsky: You can do that by giving sugar and not make him worse. I am using the susceptibility to ketosis as a criterion of severity. It is not increased

Dempsey: I was wondering whether there was any connection between heavy metal poisoning and the paradoxical effect of adrenalectomy on skin and hair growth. After adrenalectomy, most functions are impaired, but hair growth is increased and the O_2 consumption of skin is elevated. Skin and hair suggest SH groups Isn't it possible that if hair were laid down more rapidly, the pool of SH groups might be decreased and therefore heavy metal poisoning would occur more readily — that is, a given amount of heavy metal would have a greater effect than it would if the pool were normal. Conversely, to give cortisone to stop hair growth might increase the SH pool and repair the deficiency caused by heavy metals.

Ragan: In the human the hair growth is variable. In the human you lose head hair but gain body hair and it does not apply to the animal. It is a different atmosphere.

Holbrook: There is also some evidence, Dr. Dempsey, that cortisone is not the effective antisensitive agent; even in tremendous doses with ACTH it may not function by way of cortisone or cortisone-like substance. It may be a different factor entirely.

Mote: That just simply points up how little we know about the adrenal glands and adrenal corticoids.

Mirsky: We know very little about cellular responsiveness.

Mote. That is right.

Jones: I don't know why you limit it to the adrenal.

REFERENCES

1 HERRMANN, H, and PERLMANN, G E · Reaction between proteins and metaphosphoric acid *Nature* **140**, *807 (1937)*.

2 PERLMANN, G E, and HERRMANN, H · On the reaction between metaphosphoric acid and egg albumin *Biochem J* **32**, *926 (1938)*

3 PERLMANN, G E Combination of proteins and metaphosphoric acid *J Biol Chem* **137**, *707 (1941)*

4 PERLMANN, G E On the preparation of crystallized egg albumin metaphosphate *Biochem J* **32**, *931 (1938)*.

5 PERLMANN, G E Unpublished results

6 GOEBEL, W F, OLITSKY, P K, and SAENZ, A C Inactivation of biologically active proteins, and virus of western equine encephalomyelitis by periodic acid *J Exper Med* **87**, *445 (1948)*

7 GOEBEL, W F, and PERLMANN, G E · The effect of lithium periodate on crystalline bovine serum albumin *J Exper Med* **89**, *479 (1949)*

8 LINDERSTROM-LANG, K Neuere Untersuchungen uber die Anwendung von Enzymen zur Konstitutionsermittlung der Proteine *Ergebn d Physiol* **35**, *415 (1933)*

9 SVEDBERG, T, and ERIKSSON-QUENSEL, I B: Ultracentrifugal study of the action of papain on ovalbumin *JACS* **56**, *409 (1934)*

10 ANNETTS, M The digestion products formed by the action of papain on egg albumin *Biochem J* **30**, *1807 (1936)*.

11 TISELIUS, A, and ERI[illegible]
on peptic digestion of [illegible]

12 PETERMANN, M L, [illegible]
centrifugal analysis of diphtheria proteins *J. Phys Chem* **45**, *1 (1941)*

13 PETERMANN, M. L Ultracentrifugal analysis of pepsin-treated serum globulins *J. Phys Chem.* 46, *183 (1942)*

14 LONGSWORTH, L G, CANNAN, R K, and MACINNES D A An electrophoretic study of the proteins of egg white *JACS* 62, *2580 (1940)*

15 LONGSWORTH, L G A modification of the schlieren method for use in electrophoretic analysis *JACS* 61, *529 (1939)*

16 MACPHERSON, C F C, MOORE, D H, and LONGSWORTH, L G Changes in the electrophoretic composition of crystalline hen's egg albumin with age *J Biol Chem* 156, *381 (1944)*

17. LINDERSTROM-LANG, K, and OTTESEN, M A new protein from ovalbumin *Nature* 159, *807 (1947)*

18 PERLMANN, G E The electrophoretic properties of plakalbumin *JACS* 71, *1146 (1949)*

19. ABRAMSON, H A Electrokinetic phenomena VI Relationship between electric mobility, charge, and titration of protein *J Gen Physiol* 15, *575 (1932)*

20 LONGSWORTH, L G The influence of pH on the mobility and diffusion of ovalbumin *Ann N Y Acad Sc* 41, *267 (1941)*

21 EEG-LARSEN, N, and LINDERSTROM-LANG, K, and OTTESEN, M Transformation of ovalbumin into plakalbumin *Arch Biochem* 19, *340 (1948)*

22 PERLMANN, G E Electrophoretic behavior of modified oval bumins *Nature* 161, *720 (1948)*

23 LINDERSTROM-LANG, K, and OTTESEN, M Formation of plakal bumin from ovalbumin *Compt rend lab Carlsberg ser chim* 26, *403 (1949)*

24 MACHEBOEUF, M, SORENSEN, M, and SORENSEN, S P L Sur la teneur en phosphore et la solubilité de l'ovalbumine *Compt rend. lab Carlsberg* 16, *No 12 (1927)*

25 PERLMANN, G E Phosphorus content of ovalbumin and of some products of its enzymatic degradation *Nature* 164, *961 (1949)*

26 PERLMANN, G E Unpublished results

27 PERLMANN, G E Enzymatic dephosphorylation of ovalbumin and plakalbumin *Nature* 166, *870 (1950)*

28 ROSE, B, PARE, J A P, PUMP, K, and STANFORD, R L Preliminary report on adrenocorticotropic hormone (ACTH) in asthma *Canad Med Assoc J* 62, *6 (1950)*

29 DENT, C E Chromatography in the study of liver disease chromatography studies *Liver Injury* Hoffbauer, F W, Editor Trans Sixth Conf New York Josiah Macy, Jr Foundation, 1947 (p 53)

30 GYORGY, P Microbiological studies of urinary excretion of amino acids *Liver Injury* Hoffbauer, F W, editor Trans Sixth Conf New York Josiah Macy, Jr Foundation, 1947 (p 63).

31. BROWNE, J, and ROSE, B Personal communication

32 VENNING, E H Adrenal function in pregnancy *Endocrinology* **39**, *203 (1946)*

33 MARRIAN, G F, CLAYTON, B E, and SOMMERVILLE, I F · Effect of diethylstilboestrol on urinary excretion of pregnanediol and endogenous estrogen during pregnancy *Lancet* **1**, *680 (1949)*

34 KINSELL, L W, ET AL Studies in methionine metabolism III The fate of intravenously administered S^{35}-labeled-methionine in normal adult males, in patients with chronic hepatic disease, "idiopathic" hypoproteinemia and Cushing's syndrome. *J Clin Investigation* **29**, *238 (1950)*

35 WOODS, A C Clinical and experimental observation on the use of ACTH and cortisone in ocular inflammatory disease *Am J. Opth*, **33**, *1325 (1950)*

36 CLUXTON, H E Reported at the Second Clinical ACTH Conference (to be published)

EFFECT OF ACTH AND CORTISONE ON CONNECTIVE TISSUE*

CHARLES RAGAN
Department of Medicine
College of Physicians and Surgeons, Columbia University

I PLAN to talk on the effect of ACTH and cortisone on nondiseased connective tissue. I am not going to say normal. Later, if we want to, we can discuss the effect in the disease states with which we are concerned. However, I am going to present a number of figures illustrating some of the effects we have observed.

Figure 27 shows a patient with lupus who had the usual clinical response to the administration of ACTH. Shortly after starting ACTH, she developed a left parotitis which had to be incised and drained. Throughout the subsequent course on ACTH which lasted 21 days, there was no apparent granulation in this incised, drained, open, dirty wound (Figure 27). She developed a moon facies, striae, the usual hyperadrenalism. We stopped the ACTH and within four days granulations appeared as shown in Figure 27. Another patient in whom all the criteria of lupus were present, and who was treated with large doses of ACTH is shown in Figure 28. She had been extremely ill for a month preceding admission. She had delivered and the episiotomy wound made at the time of delivery was open. It was not epithelialized but contained large granulations at the time she was admitted to our service. Three days after she reached us a decubitus ulcer broke down. This pressure sore did not show any evidence of granulations nor was there any evidence of repair. This patient died of pulmonary emboli. We also had a patient with dermatomyositis in whom we did a muscle biopsy and the wound, while on ACTH,

*This work was done in conjunction with Drs J Wallace Blunt, Edward L Howes, Raffaele Lattes, Karl Meyer, Charles M Plotz Morton C. Creditor, Margaret Bevans, and William L.

FIGURE 27 * Observations made during the course of a case of lupus erythematosus disseminatus A.A. indicates acetylsalicylic acid

*Figures 27 and 28 reprinted from article by PLOTZ, C. M., BLUNT, J. W., and RAGAN, C.: Effect of pituitary adrenocorticotropic hormone (ACTH) on disseminated lupus erythematoses. *Arch. Derm. & Syph.* 61, 913 (1950)

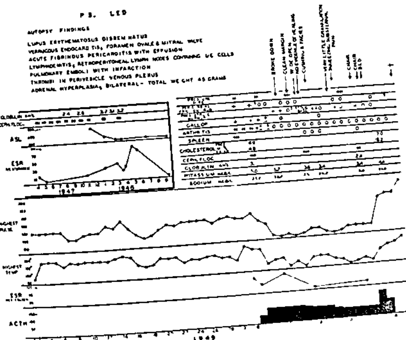


FIGURE 28 Observations made during the course of a case of lupus erythematosus disseminatus *A.S.L.* indicates the antistreptolysin titer

required 12 days before the surgeon would remove the sutures After the cessation of ACTH, he was willing to remove the sutures in four days.

In a patient with periarteritis nodosa a nodule was removed before ACTH therapy; at the end of 7 days, while still on ACTH, ... Figure 29 shows that ... was some

... in whom

Figure 30 shows a patient with ... this same procedure was carried out A biopsy was made, a small incision of the skin and subepithelial layers, and ACTH was started. Seven days later, while on ACTH, the wound was again biopsied; there was very little evidence of fibroplasia but in some sections there was fairly good epithelialization across this wound.

FIGURE 29 One-week-old skin wound from patient on ACTH Hematoxylin and eosin, X11

FIGURE 30 One-week-old skin wound from patient on ACTH Hematoxylin and eosin, X 38

Figures 29, 30 and 31 reprinted from CREDITOR, M. C., ET AL Effect of ACTH on wound healing in the human *Proc Soc Exper Biol & Med* 74 245, 246, 247 (1950)



FIGURE 31 One-week-old incised wound one week after cessation of ACTH Hematoxylin and eosin, X 39

In Figure 31 is shown the same patient 7 days after ACTH was stopped, and you can see that fibroplasia was present ... there was an abscess in the depths of the wound.

We then turned to animals, rabbits heavily treated with ...sone and markedly hyperadrenal, and used several accepted ...niques for the study of wound healing. These animals ... 25 mg. of cortisone in two doses daily for three days ...

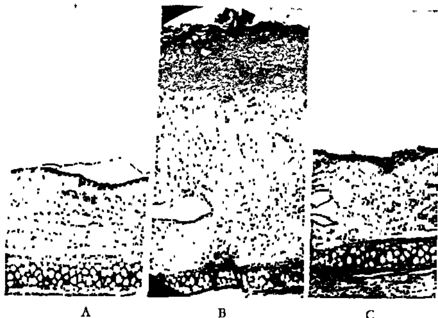


FIGURE 32

A—Cross-section of normal rabbit's ear—left

B—8-day granulating wound control—center

C—8-day granulating wound, animal treated with cortisone—right

Note thickness of connective tissue and the cell density in the normal ear In the control wound the granulations are higher There are many fibroblasts with collagen fibrils and many capillaries are seen. Two old large dilated blood vessels are present The cortisone granulations are not much thicker than the normal connective tissue of the ear and all the old large blood vessels are dilated Capillaries are present However, cell concentration, particularly of the round type and those resembling fibroblasts, is increased These cells show few signs of increased proliferation

wound was made and subsequently throughout the experimental period. These wounds were made by the accepted technique used by Dr. Howes in which the skin and the subcutaneous tissue were peeled off, leaving epithelial islands in the middle. In the treated animals, after five days, the vessels which were present at the time the wound was made were still visible, whereas in the untreated controls granulation tissue was evident. Eight days after the wound was made a similar situation existed where the blood vessels were still apparent in the cortisone treated animal while in the control the wound showed many granulations. In both instances, there was some epithelial growth from the side of the wound, apparently more marked in the control animals.

Figure 32 is a microscopic section showing a typical picture. (A) shows the normal rabbit's ear before operation. (B) shows

*Reprinted from *Bull N Y Acad. Med* 76, 251 (1950).

the control animal ear and new appearing granulations are apparent. (C) shows the treated rabbit which received 25 mg of cortisone a day, which is equivalent, may I say, to 10 mg. of cortisone per kilo of rabbit. With special techniques, it may be shown that there is less metachromasia in the treated animal, the fibroblasts look smaller, more pyknic, less swollen, and there are fewer new capillaries. Thus, in the treated animal, the three elements—fibroplasia, capillary growth and ground substance proliferation—were depressed.

Some experiments were performed on the expansile strength of stomach wounds in the rat. This technique is a fairly well-established one in which the stomach is removed seven to eight days postoperatively, the duodenal end is tied off and the cardiac end is inserted into a catheter connected to a manometer. Increasing pressure of gas is introduced through the cardiac end which is recorded until the wound disrupts Table IV shows the pressures in millimeters of mercury necessary to break the wounds In the controls you can see there was a wide spread with an average of 111 mm. With 5 mg. of cortisone, which is equivalent to 20 mg., per kilo there is no significant difference between the control and treated animals. At 10 mg. a significant difference begins to appear. In other words, the rat needs eight to ten times as much cortisone on a mg. per kg. basis to achieve this effect as does the rabbit. This same type of dosage differential has been found to apply to other experiments in rats. One technique was to remove a piece of rat rectus muscle and then sew up the skin wound. In the control animals, this was filled with an area of granulations in

TABLE IV

Summary of Findings: Rat Stomach Inflation
(Pressure in mm Hg necessary to break wound)

	Controls	10 mg Cortisone o d	5 mg Cortisone o d
	98	58	96
	99	66	104
	112	94	70
	134	84	114
		70	92
		76	
Average—	111	75	95

a period of seven to eight days, whereas in the treated animals the defect remained open. In guinea pigs there was approximately the same dosage requirement of cortisone as in rats. Spain at Madison, Wisconsin, last week described his work with wounds in mice and in these wounds granulations were inhibited by a dose of 2 mg. a day which is equivalent to about 100 mg. per kilo. We have had no experience with mice but I mention Spain's work primarily because he did supply adequate amounts of ascorbic acid and found that this did not modify the healing process or the inhibition of healing during the hyperadrenal state.

A great deal of our work was done with what we hoped was a closed wound. This represents a fracture in the rabbit femur done by the method of Clay Ray Murray, in which an anesthetized rabbit receives a blow on the femur, breaking the femur and then is allowed to stay in his cage without splinting for the experimental period. For two days these animals again were pretreated with 15 mg. of cortisone a day, equivalent to 5 mg. to 6 mg. per kilogram. The control animal and the cortisone-treated animal, two days following the fracture, showed little difference grossly. At four days the difference became apparent in the sense that in the control animal there was considerable resorption of the hemorrhage and beginning callus formation. At six days the difference was more apparent. The callus in the control



FIGURE 33A Section through comparable fracture site, 8th post-fracture day, cortisone-treated rabbit, 76 X, hematoxylin-chlorazol fast pink B The entire healing zone is represented by the relatively narrow area on the right. No clearly defined cartilage and no osteoid can be seen.



FIGURE 33B * Section through fracture site, 8th post fracture day control rabbit, 76 X, hematoxylin chlorazol fast pink B At the top, below the margin of injury, is a dense area of fibroplasia There is a wide area of well differentiated cartilage below that and at the bottom osteoid with bone trabeculation is noted

* Figures 33A and 33B reprinted from article by B[illegible] J. B. et al. Effects of cortisone on experimental fractures in the rabbit. Proc. Soc. Exper. Biol. & Med. 73, [illegible] [illegible]

animal was readily apparent. In the cortisone-treated animal, hemorrhage was still present. At eight days there was a small amount of callus present in the treated animal (33A) while the hemorrhage was still not completely absorbed; whereas in the control animal (33B) there was a large amount of healing callus.

In summary, the effect of hyperadrenalism on the healing of fractures can be stated simply as follows: up to the fourth day there was very little difference between the control and the treated animal microscopically. In both the control and treated animals there was metachromasia. There was some fibroplasia, but from the fourth day on, the difference became quite apparent in the sense that there was a marked increase in fibroplasia in the control animal, and in a few instances beginning formation of cartilage. Whereas in the treated animal at the fifth day the fibroplasia was much less We were unable to see any cartilage until the eighth day, when a small segment of cartilage was visible in the treated animal. In Figure 33—the eight-day fractures—in the control animal (33B) there was some fibroplasia, a large area of cartilage and some osteoid formation, whereas in the treated animal the fibroplasia was about the only differentiation that took place, and the large empty space contained the hematoma which came out on fixation (33A).

In a Masson's trichrome stain, at the eighth day, the difference was quite striking. Osteoid formation was present in the eight day control and metachromasia was still present whereas in the eight-day treated animal the metachromasia was all in osteoid and in the cartilage but the metachromasia here continued to be diffuse. This is different from what we have seen in the incised wound and in open granulating wounds where there was complete suppression of all elements up to eight days following the wound.

Angevine. Yesterday the question was raised about the possibility of fibrinoid occurring in normal healing areas. Such a pink area as that was shown originally by Dawson (1) to be fairly typical for fibrinoid with Masson stain. It looks like fibrinoid on that slide. Would you agree, Dr. Bennett?

Bennett· Yes.

Ragan: I would like to talk about incised wounds in rabbits. Incised wounds followed somewhat the pattern of open granulating wounds in the sense that there was decreased fibroplasia and decreased metachromasia. In a trichrome stain at times in these incised wounds we were unable to see the incision on section. These wounds were all tightly sutured with either silk or clips. At times we needed polarized light to see the incision because there was apparently no change in the continuity of the collagen fibers in

the best microscopic light examination. There was some epithelialization and as I will show you subsequently, any area of fibroplasia apparent was in the subepithelial layer. With toluidine blue stains, in the control animal at seven days there was good evidence of fibroplasia.

Ultimately these wounds will heal, and it was a question in our minds whether there was a local escape, allowing healing or whether the animal itself became insensitive to the action of the hyperadrenal state whereby healing took place. So we next did two wounds at different times in the same animal. An incised wound biopsied fourteen days after the wound was made in a control rabbit showed a wide area of fibroplasia with good healing and relatively little metachromasia at the time of biopsy. Rabbits were pretreated with cortisone for three days and then a wound was made The cortisone was continued and nine days later another wound was made and again the cortisone was continued for five more days. Thus in the same cortisone-treated rabbit a five-day and a fourteen-day wound were present. In the five-day wound there was relatively little evidence of healing. In the fourteen-day wound in the same animal there was good healing So if there was any effect, it was a local one at the tissue site.

The more hyperadrenal you make the animal the more inhibition of connective tissue. At 10 mg. per kilo per day of cortisone in the rabbit, inhibition of *connective tissue persisted for a considerable* length of time. At 2 mg. per kilo per rabbit at about eight days there was apparent escape in the sense that there was the beginning of good granulations appearing in the wounds. There was a considerable difference in species as far as the sensitivity to the given dose of cortisone goes, and this was not wholly a question of the size of the animal. It is apparently well known that small animals require relatively larger amounts of any kind of preparation per kilogram than do larger animals, but the dog at 7½ mg. per kilo did not develop glycogen deposition of the liver, and we were unable to see any effect on wound healing in a dog at this dose.

Holbrook: What dose was that?

Ragan: Seven and a half milligrams per kilo When a rabbit is treated with very large amounts of cortisone to achieve an effect on wound healing, large amounts of glycogen can be shown to be deposited in the liver by the periodic acid Schiff reaction.

Since we are supposed to tell what problems we have come upon, I thought I would mention one that bothers us considerably, and that is, with large doses of cortisone our animals are not too

happy. Undoubtedly in the rabbit house we have an endemic diarrhea, and of the rabbits in adjacent cages, one control and one cortisone treated, the cortisone-treated one will much more likely develop diarrhea. However, at smaller doses of cortisone, the rabbits during the period of time we have used them, which in most cases is not longer than eight days, seem to maintain their status as healthy animals, but if you go up to two or three weeks with larger doses of cortisone you have great difficulty in keeping your rabbits alive.

Fremont-Smith. They are more susceptible to infection?

Ragan: That is what I was going to bring up later on, Dr. Fremont-Smith.

The humans in whom we have seen this change in wound healing, were obviously not in ill health. They were feeling better than they had felt in a long while, so I don't think this effect is completely one of sick rabbits. Certainly a sick rabbit will heal less well than a well rabbit.

As far as the mechanism goes, we have reached a point beyond which we cannot proceed. We feel definitely that there is a delay in connective tissue growth but not a complete suppression of connective tissue growth. Incised wounds will ultimately heal, and fractures also.

In open wounds with granulations, epithelium will ultimately cross, but at a lower level of granulation The difference between the incised wounds and the granulating wounds on the one hand and the fractures on the other is difficult to explain. Our theories explaining these differences do not warrant mentioning at this time.

The effect of cortisone is probably a local effect. Baker mentioned this at the AAAS Conference and Dr. Howes (2) at our institution has had some experience with the local application of cortisone to granulating wounds in ears—

Meyer: What do you mean by "local effect?"

Ragan: If you just rub cortisone on a granulating wound, it does not granulate

Fremont-Smith: Not enough for absorption.

Ragan: The other ear does not do it. Dr. Meyer will have something to say about that later.

Mirsky: Do you give your material systemically?

Ragan: Yes.

How do you explain this effect if it is a local one? The hypothesis is that the tissue is unable to utilize some material during the hyperadrenal state, until the concentration builds up to a certain

point, and beyond that concentration healing does take place.

We have injected several things into these wounds in an attempt to supply an excess of some material. So far nothing we have injected has had any change in the effect. I would like to say one thing, though, that we did inject hyaluronidase and grossly good healing of the wound took place since the wound could not be pulled apart. When we sectioned these wounds we saw that it was simply fibrin which was markedly cohesive, and there was no evidence of fibroplasia.

In some way this looks like scurvy. In other ways it does not. Certainly, there is no evidence of bleeding in these animals, and Spain's work at least shows by administration of large amounts of ascorbic acid to mice that he was not able to change the effect. However, this does not mitigate the fact that you may have a local defect in utilization of ascorbic acid.

Mote: It seems to me that we are going to see several schools of thought develop with respect to tissue repair. I think Dr Ragan has one point of view, John Browne has another one, and perhaps George Thorne is sort of betwixt and between. Certainly some investigators working at the human level have not had the same experience in their estimation that Dr. Ragan has, like one patient with lupus in Chicago had an abscess that would not granulate before she was on ACTH, but when she was put on ACTH it granulated right in.

Holbrook: This is cortisone.

Mote. No. ACTH.

Ragan: I am talking about ACTH in humans.

Holbrook· All right.

Mote: Then there are at least two other cases with appendicitis who were operated upon and as far as one could tell they healed perfectly well. Of course, John Browne does have several hypotheses which are in some conflict. My own personal point of view is that many more of us would be dead if this were really true to the nth degree. On the other hand, most wounds do heal whether from major surgery or otherwise. There is definite evidence of endogenous adrenal cortical stimulation in these situations. It is difficult for me to see that, even with ordinary stimulation of the adrenal gland, you can have any marked inhibition of healing in the light of statistical results of surgical operations. I would like to get some other ideas on that.

Ragan: May I say one thing first? We have never implied that healing does not take place. It does ultimately. All I can answer those people who say that they heal is, did they biopsy their

wounds, because healing is delayed in the wounds we have seen.

Angevine. I think the important thing is not so much what the effect is on the healing of wounds as how it affects the fundamental process of fibrillogenesis that we were talking of yesterday. That is where attention should be focused because irrespective of whether the wounds heal in patients, a change has been produced in animals. It seems to me what now concerns us is the mechanism involved.

Mote: I would agree that getting at that mechanism is the basic problem. You are working at a pretty high concentration of corticoid. Whether a smaller amount might mobilize protein and cause the connective tissue proliferation is thoroughly possible in my view.

Bennett· In looking at your preparations I could not help being impressed with the lack of any cellular proliferative activity initially. This is entirely different from the impairment of healing in ascorbic acid deficiency. It appeared as if these incised wounds were unchanged even after many days from time of injury. Is that true?

Ragan. That is true. In markedly hyperadrenal animals when you give large doses of cortisone there is no invasion of macrophages

Fremont-Smith: What about the polys, or don't you get them first?

Ragan: You don't. We note that during hyperadrenalism there is a delay in the appearance of new fibroblasts. Once fibroblasts are laid down, their appearance is not modified during the time of treatment we have used—15 days. The hypothesis may then be raised that the cell inhibited may be the prefibroblast—a cell similar to the macrophage.

Meyer: Dr. Ira Jones and I (3) did some experiments on the local effect of cortisone on the vascularization of the rabbit cornea. We injected both corneas of groups of 6 rabbits with 0.05 cc. of 0.2 to 0.5 N NaOH to induce vascularization. One eye was injected subconjunctively with 0.1 cc. of cortisone acetate or in one group with cholesterol in the same vehicle supplied to us by Merck. The other eye served as a control. Injections were made in the first and third day. After about three days the blood vessels started to invade the cornea. The number of blood vessels in the control eye were very much more numerous than in the cortisone-treated eyes; in the first series, for example, 170 as compared to 10 for the treated eyes. Some vascularization takes place in the control eyes, too, depending on the severity of the alkali burn. The

capillaries finally recede leaving a leucoma By the way, we used a penicillin ointment on the first day to prevent infection of the burn. We assume that there is a chemotactic influence on the marginal capillaries of the sclera in these burns which leads to the sprouting of these capillaries and the invasion of the cornea. Years ago we proposed that the mucopolysaccharide of the *substantia propria* of the cornea was responsible for the absence of vascularization of the cornea. Hughes in Friedenwald's laboratory (4) had shown that alkali burns led to a rapid loss of metachromasia of the cornea. We showed that in about one hour after alkali burns, the cornea loses polysaccharide as shown by a loss in hexosamine, presumably by enzymatic breakdown. The mucopolysaccharide of the cornea is a sulfate ester which is digested by hyaluronidase and, furthermore, the enzyme exists in the normal cornea. By the way, sulfate esters, in general, may have something to do with absence of blood vessels as in hyaline cartilage and amyloid The Clarks in Philadelphia (5) did a beautiful experiment on the suppression of capillary growth by pieces of cartilage in the transparent chambers in the ears of rabbits We proposed the hypothesis that removal of the sulfated mucopolysaccharides permits capillary growth. Assuming this to be true, then the question arises whether the split products of these polysaccharides are growth stimulators for the endothelial cells.

Gyorgy: What does cortisone do; how does it prevent it?

Meyer: It may prevent the reactivity of the endothelial cells or may remove the split products in some way This is frank speculation.

Zweifach: It is my impression that Clark (6), in his classical studies on the ingrowth of blood vessels into a chamber in the rabbit's ear, found that endothelial sprouts growing into the non-vascular center of the ear chamber were usually accompanied by fibroblasts. In fact, they point out that the fibroblast growth usually occurs just ahead of the endothelial growth. Thus, there may be an interrelationship between adequate vascularization of an area and the proliferation of fibroblasts

Meyer: I think that this does not occur in the cornea

Zweifach: It is possible that there are already present sufficient preformed structures in the cornea along which the endothelial sprouts can enter.

Ragan: In our open wounds in rabbit ears in the lowest dose of cortisone that we have used the first appearance of fibroblasts is around old blood vessels. These fibroblasts grow between blood vessels and then granulations appear.

Zweifach: It is not clear whether the ingrowing fibroblasts soften the connective tissue so that the ingrowing endothelium can then enter more readily or whether the endothelial sprouts require some organized structure onto which the advancing endothelial cells can attach themselves.

In our experience, using the Essex chamber, at early stages nothing is apparent but blood vessels, but we are certainly not facile in this technique.

Meyer: I believe the ophthalmologists who have studied the vascularization of the cornea believe that the sprouting of endothelial cells leads to new formation of capillaries.

Zweifach: I do not wish to convey the impression that the new formation of capillaries occurs from fibroblasts. All the evidence indicates that endothelial sprouts develop only from preexisting vascular structures. I merely wanted to bring attention to the possible interrelationship, either chemical or physical, between the ingrowth of fibroblasts and the rapid advance of newly formed capillaries into an area.

Ragan: There is a great difference of opinion among surgeons dealing with wound healing, whether you need capillary sprouts, ground substance or what. All we can say from our experience, as far as we can tell every element is depressed in the open granulating wounds.

Holbrook: Dr. Ragan, may I go back a minute? Do you know how much information is available on wound healing in Cushing's disease?

Ragan: I have one Figure I can show you.

Holbrook: All right.

Mote: I think it may be entirely a question of degree, in that excessive corticoids seriously alter cellular physiology and nutrition and thereby affect growth. At low levels of corticoids it is possible in my view to "jar loose" as John Browne says, a lot of amino acids which would be available for tissue repair and proliferation.

Ragan: Figure 34 shows a boy with naturally occurring Cushing's disease and I think there is little doubt but that he had Cushing's disease.

Figure 35 shows the result of an incision for exploration of adrenal tumor. This is our most severe example of poor wound healing in Cushing's. We can see he had a large open infected wound there and in our 34 cases of Cushing's disease we have seen evidence of poor wound healing in a few. Again may I refer back to my statement to Dr. Mote? No biopsies were made of these wounds. So we don't know. They ultimately healed in most of them.



FIGURE 34 Cushing's syndrome

FIGURE 35 Abdominal wall—left pre operative, right post-operative, showing widespread inflammatory infection without localization and poor wound healing

Fremont-Smith: How long after the operation was this one?

Ragan: Eleven days and he died three days later. This turned out to be an infection with a micro-aerophilic hemolytic streptococcus. We have several instances in Cushing's of local infection. In one instance a woman recently died who had a small infection on her nasal septum and from this a hemolytic streptococcic septicemia with pericarditis developed from which she recovered with antibiotics. In one of our patients with rheumatoid arthritis treated for a long period of time, with ACTH, there was present a concomitant diverticulitis. A diverticular abscess developed in the sigmoid. She had some abdominal pain of never great severity and we were not aware that an abscess had developed. We were not aware until she started to pass fecal matter per vagina and ultimately we were able to demonstrate a fistulous tract between the bladder and the sigmoid. At the present time she has a colostomy as a shortcircuiting measure. We looked upon it as a manifestation of poor localization of long-continued infection in long-continued induced hyperadrenalism. This is again speculation and we are not certain of the cause and effect relationship. The only other evidence we have is very indirect evidence in that the rabbits made hyperadrenal develop rabbit dysentery more readily than do the controls.

Holbrook: Would you say that again?

Ragan: The rabbit, made hyperadrenal in the same room with the control, develops dysentery, whereas the controls do not.

Mirsky: Were the cases in whom you first observed the decrease in rate of healing receiving doses of cortisone or ACTH which were effective therapeutically?

Ragan: Yes, sir.

Mirsky: I am going back to Dr. Holbrook's observations of an increase of histidine excretion in the pregnant woman on the one hand, and the effect of ACTH, on the other. Then, if the two are related, they are due to the same steroids which are therapeutically effective in the patient with rheumatoid arthritis. Then the question comes up as to whether there is any evidence [illegible] wound healing during pregnancy.

Ragan: I have not been able to find that out yet, Dr. Mirsky.

Mote: I think I should make a point and that is simply look at this world as it is and wonder why we don't all die of infection and we don't all die of trauma. It still leads me back to my personal belief that the adrenal cortex plays a definite role with respect to health and disease in general.

Mirsky: I must interrupt. That does not mean that that state-

ment is a fact. That we all survive does not mean the adrenal cortex is responsible.

Mote: I realize that, but I believe there is a fair amount of evidence that we all do have stress phenomena and most of us survive these stress phenomena quite well, and that to my view is at least to some extent evidence that the adrenal gland plays some protective role for the individual. On the other hand, I recognize that there is evidence that, overstimulation or administering too many corticoids adversely affects many processes in the body. In this general connection I would like to know from Dr. Holbrook what his opinion is of the effect of an operation in a rheumatoid arthritis patient. How long do they stay in remission after a major operation?

Holbrook: That is very variable. Some of them, of course, get no remission at all. I think the longest one I have seen is three months.

Ragan: Some do not, and some have a remission for two or three days.

Holbrook: It is a wholly variable procedure.

Ragan: After a major operation You must admit you have not made them hyperadrenal enough to get a longer remission

Mote: In other words a variation in the magnitude and duration of endogenous ACTH stimulation of the adrenal cortex as a consequence of trauma.

Holbrook: Do you have in mind, Dr. Ragan, doing any more work of this kind on minimal effective dose to see whether the effects—the healing time I should say—would be different? I should think that might be very interesting.

Ragan: What we have hoped is that by reducing the dose of cortisone to the least possible effect we would see which element appeared first because that is what we wanted to know If we could see what element appeared first, we would have some idea what we were inhibiting, but using the rabbit's ear with 2 mg. per kilo per day the fibroblasts grow out from the old blood vessels, and you see fairly decent granulations appearing from the old blood vessels.

Holbrook: Cortisone is not as satisfactory

Ragan: In animals it is very satisfactory because we have not many nurses to stay up all night with animals.

Holbrook: It is not the substance to test minimal effective doses, that is my point. Have you by any chance put any ACTH on that wound locally to see whether it had any effect?

Ragan: No, we did not do that.

We are now studying embryonic tissue. If the chick embryo is a decent subject to work with, it is a lot simpler. There, again, we have to go back to find out what the normal anatomy of the chick embryo is; very few clinical pathologists know much about it. I know nothing about it. We have developed a hypothesis for this, for what it is worth. If you presume that in these diseases of the connective tissue you have a turnover phenomenon going on all the time where, as Dr. Dempsey said last night, he believes—and I think most of us believe—there is a continual replacement of this tissue, if you interfere with that turnover in some way you may cause a defervescence of symptoms of these diseases. That is our hypothesis. In a mildly hyperadrenal state in a rheumatoid you have delayed the reaction time of the connective tissues to the point where it does not respond to the trauma, whatever it is that initiates rheumatoid arthritis. When it does not respond, the patients do not have symptoms. This would certainly avoid the issue which is raised by the fact that in these wounds they do ultimately heal. You have not suppressed the activity of the connective tissue completely, which is what I want to stress. This is not a complete suppression of activity. It is merely a delay in healing or a delay in reaction time. That is a wild hypothesis. It may be so, and it may not. It is what we are working on at the present time.

Holbrook: I think that is a very interesting report.

Bennett: Is there any information available on the effects of cortisone or ACTH on tissues from rheumatoid arthritis patients? We have had only one set of biopsies of synovial tissues. These indicated a striking diminution in the intensity of inflammation, including edema and hyperemia, withn two weeks after administration of cortisone.

Holbrook: We have done a fair number of routine deltoid muscle biopsies but have found them very unsatisfactory from the standpoint of following recovery. The same criticism perhaps exists as has been made with regard to synovial membrane biopsies. It is always impossible to examine exactly the same tissue before and after treatment as even a centimeter's distance away the tissue may appear quite different.

Bennett: We have had only one pair of synovial biopsies.

Holbrook: We have not done those because we thought the chance there, where the local process was going on, was less good in getting an identical piece than in the muscle.

Angevine: We have examined a few skin biopsies from cases of disseminated lupus, erythema nodosum, scleroderma and amyloid, before and after treatment with ACTH and, as yet, have noted no significant difference histologically.

Dempsey. I can conceive of cortisone having an effect on both the epidermis and subcutaneous connective tissues. In one species the effect might be quantitatively more apparent in one place than in another, while in another species the sensitivity might be reversed. As to the embryological aspects of this, I believe the connective tissues develop and grow before the adrenal does. Mesenchyme puts in appearance before the adrenal cortex. Certainly the adrenal cortex does not have much to do with the embryological formation of mesenchyme and similarly from a phylogenetic point of view connective tissue puts in an appearance in animals lower than those which have an adrenal cortex. On the other hand, I was interested in what Dr. Ragan had to say about the working hypothesis. I was reminded of the general statement in endocrinology that no hormone initiates, *de novo*, any known effect, but only accelerates or inhibits effects which go on whether or not the hormone is there.

Thyroid, for example, can fix iodine and manufacture thyroxin in the complete absence of the pituitary gland. It just does it faster when the pituitary is present. It seems to me it is asking too much of the adrenal cortex to expect it to control absolutely the growth and development and differentiation of the connective tissues. Indeed, the demonstration of an altered rate of formation of connective tissues, is itself remarkable. It is, however, a phenomenon consisting of accelerating an already occurring reaction.

Gyorgy: If I may refer to a possible practical application of Dr. Ragan's observations, one of the most tragic diseases in premature infants, with a birth weight under 3-4 lbs., is the so-called retrolental fibroplasia. The condition is based on excessive growth of retinal capillaries and of fibrous tissue behind the lens and leads almost invariably to blindness. In a few early cases ACTH appeared to stop further growth of fibrous tissue. However, in further observations, this beneficial effect proved to be only temporary and in some instances was not demonstrable at all. Although the number of observations is limited, ACTH in this condition appears to be not too promising.

Angevine: Does anybody have any observations on keloids?

Mote: No.

György: I may add that we are planning to implant cortisone pellets in the spleen of children with severe diffuse hepatic fibrosis.

Mote: Either one or the other and the actual result may depend in part on the general and tissue nutritional status and the magnitude of the adrenal cortical stimulus.

Mirsky: Yes. The question that then arises is whether the observers are dealing with the same phenomenon, with the same conditions.

Mote: They are not dealing obviously with the same conditions. For one reason, you would be killing human beings if you worked at the level of dosage that Dr. Ragan has been working with in animals.

Mirsky: Talk about humans. Let us stick to man.

Mote: Just on the human level there is a difference of opinion.

Holbrook: Due to dosage maybe.

Mirsky: Therapeutic dosage.

Mote: The question is dosage. Take the case of lupus that had a sterile abscess before being put on ACTH; it had opened and would not granulate. It took 150 mg of ACTH to get the temperature down to normal and yet within a matter of a few days that wound started granulating and healed all the way in on *that dosage*. That *is* just an example. In most of the situations outside of lupus the doses have not been as large; 40 mg. to 50 mg. of ACTH is more common, which is not very strenuous metabolically. It may be partly a question of dosage.

Ragan: The figures showing incised wounds were made on 40 mg. of ACTH.

Mote. Keep in mind, I said either one or the other can happen in the estimation of the people who have done biopsies and surgery

Mirsky: I want to get to that point. I assume then, that you mean that with the same therapeutically effective, relatively low dosages you can have two sets of observations.

Mote: Yes.

Mirsky: That raises a question as to whether all the other conditions involved were also the same. After all, dosage and therapeutic remission are not the only two conditions we are dealing with.

Holbrook: Different individuals?

Mirsky: Let us assume the same

Mote: All right, you have John Browne who expresses the opinion that it is a question of nutritional status, not only general but specific in a tissue area that may enter in the healing or nonhealing.

Mirsky: The reason I raise this question is that Ferris and I have been playing with the problem of dissociating the various effects

of ACTH in the individual subject. We have some evidence that on one type of regimen one can get one response, and that the same individual on another regimen, but receiving the same dosage, will show another response. For instance, the influence of high and low sodium intake on the response to ACTH.

Mote: That raises a broad problem: what was the metabolic experience of the patient previous to the time at which you started to do something.

Mirsky. I am emphasizing that all conditions may be standardized.

Fremont-Smith: We have other examples. Pitressen, for example, was first described as a diuretic—it is now known as an antidiuretic. Why? Because the first experiments were done in animals under anesthesia. *In animals under anesthesia, pitressin is diuretic,* but when you give pitressin to nonanesthetized animals it inhibits water diuresis. To go one step further, pitressin becomes diuretic in unanesthetized animals provided you have dehydrated them *beforehand. You get the basic principle that the response of the* organism to stimulus is as much dependent upon the state of the organism as upon the stimulus. The old concept that a single stimulus always gave the same response is false. We have been thinking this afternoon in terms of a single response to a given stimulus and feeling outraged that the same stimulus could give different responses

Mote: In all the ACTH studies—I think Dr. Ragan would agree to that—everyone bumps into what has been called the parodoxical effect, even in the same individual on the same dose and the same regime.

Meyer: Dr. Ragan's demonstration of the response to the 5-day wound and to the 14-day wounds on the same animal, with the same nutritional environment, internally as well as externally, would show that.

Jones: This discussion distresses me greatly. It seems not to be a question of single stimulus. It is a question of knowing actually the truth about what produces or fails to produce the response in question, or the condition that you are trying to find. That means you just don't know the whole answer.

Meyer: That is true.

Mote: We don't know enough about metabolism.

Meyer: This is not purely a problem of cell growth.

Mote: No.

Jones: It has many facets.

Mote: I am speaking metabolically, physiologically, and of the whole picture. You do have wide variables of response in the same individual on the same dose at different times.

Mirsky: Start with a high sodium intake, give ACTH and desalt the moment you start treating the patient. Even though we kept all external conditions constant we did not have the same nutritional status.

Holbrook: Of course you did not.

Mirsky: So the control of that type of experimention is extremely difficult.

Fremont-Smith. What we need is specification of the differences. We must not be surprised that there may be differences. We must specify our areas of ignorance.

Mote: They are quite large.

Fremont-Smith· They are very large but they still need to be specified

Mote: Oh, yes.

Fremont-Smith We will gain more by specifying rather than by giving a broad sweep.

Mote: Take this problem of electrolytes for instance; the more that is really studied, going back and resetting the conditions of the experiments, the more we are sure that what at first looked like bizarre results will appear to make some sense at some stage.

Mirsky: I can exemplify with another experience. We all know that patients given ACTH may develop a variety of psychological symptoms. I am thinking of frank psychoses. We now know that some patients may have their psychoses almost eliminated by the administration of potassium even though we did not have any evidence that there was a real disturbance of potassium metabolism

Mote: That is one of the type of experiments that I think certainly poses this problem.

Mirsky. There will be a dynamic aspect to that.

Holbrook: I think it is perfectly clear to all of us who have worked with these things and studied them that there are relatively few things that you can predict with certainty which will happen to any given patient under a given state of conditions. I tried to point that out a while ago when I mentioned the rising eosinophil count, the stable sedimentation rate and all these other things but they are different individuals and they have a different functioning capacity to their adrenal cortex. They respond differently than other people who look exactly alike. Two people can be right together. You cannot tell them apart from all the control studied. You don't know which one when you start to administer one of

these substances will produce the bizarre effect and which one won't. You cannot predict it.

György: Of course, ACTH will produce quite a number of corticoids whereas with cortisone you probably just give one Therefore, one has to distinguish between these two types of medication, too.

Mirsky. We don't know that today.

György. Certainly one should consider it.

Mirsky: We don't know whether that one steroid you are giving will be converted.

György: You have less complicating conditions than with ACTH.

Dempsey: ACTH may cause the production of more than one steroid.

Gyorgy: That is certain. For cortisone you just assume.

Mirsky. It does one thing in one man and another in another.

Dempsey: There are about 26 different steroids in the adrenal.

Mote: You don't know how many of the 26 are there at any given time.

Mirsky: In talking about the problem of responsiveness of the cell, I am thinking about Ingle's work (7) where he gave stilbestrol to rats and produced a marked glycosuria. If he removed the adrenals he got no response to stilbestrol. Subsequently he found that if he gave the adrenalectomized rat just enough adrenal cortical hormones for maintenance and then gave stilbestrol, glycosuria ensued. Experiments of this sort indicate that the cell required a minimal quantity of hormone to enable it to respond to a variety of stimuli. Perhaps we are dealing with essentially the same situation in Dr. Ragan's experiment, where cortisone makes the cell respond to the toxic reagent in one state and to an entirely different type of stimulus in other situations.

Mote· That is one of the major points. There is no question about it or Dr. Ragan's experiments. But just how the human or animal body overall is going to fit in the infinite number of situations and reactions or how it is going to react to them is pretty problematical all the way through.

Mirsky. I have one fear these days, a fear with reference to the concept of the primary role of the adrenal. We must not forget that the adrenal is not the only adaptive mechanism utilized by the organism; it isn't the only gland available for adaptations, even though it is one of the mechanisms which are important for total adaptation.

Mote: I thoroughly agree there. I think one of the biggest headaches of the next decade is the group of hormonal interrelationships and the compensating mechanisms between them

Dempsey. I would like to support what Dr. Mirsky said about the necessity of knowing more about end organ response. Speaking as an endocrinologist, I should say we know very little about end

[illegible]

and an increased or decreased end organ responsiveness For example, in even such a simple endocrinological operation as assaying an unknown estrogen, we must deal with this problem. The rat unit is defined as the amount of estrogen which will induce estrus in 50 percent of a group of test rats Needless to say, the rats will be as identical as can be contrived by the investigator Why is not estrus produced in all of the rats? Obviously, one animal has a different sensitivity than another animal does. So in all of these matters of end organ sensitivity we are dealing with a statistical rather than with an individual result It seems to me that one of the largest unplowed fields in endocrinology today lies in this area of end organ responsiveness. The factors which regulate the end organ are adaptive mechanisms just as much as are the hormones themselves.

Mirsky: I think it is. We know, for example, that the responsiveness of the cell can be influenced by sympathectomy I use the word "responsiveness" because I do not want to implicate any kind of mechanism Thus, one can increase the response of some tissues to humoral agents by denervation. There is also some evidence that suggests that stimulation of the sympathetic nerve supply to the cell may decrease the responsiveness to humoral agents We are very much interested in learning what the conditions are that change the responsiveness of the cell to a humoral agent. There is some evidence that suggests that some patients with diabetes have no decrease in the amount of insulin which is produced by the pancreas but that there is a change in the amount which they are utilizing or a change in the responsiveness of the cell, or a variety of changes in the reactions of the enzyme systems within the cell to the humoral factor. I believe it is exceedingly important to bear that in mind, and I am glad you raised that question

Fremont-Smith: In that connection the kind of method that Dr. Ephraim Shorr has used with the vaginal smear in testing the

full response of the patient to estrogen administration might be helpful. They are not depending upon rat unit but are using a method of getting full response in the individual patient using the individual as his own basis for measuring when a full therapeutic response has been achieved.

Mirsky: You are touching upon a very serious problem. I remember some unpublished experiments which we did. We conditioned rats to jump across a stand. Vaginal smears were made daily. When the rats were frustrated, they went into anestrus. Suppose a rat were to be frustrated by his neighbor in another cage and you then use that animal for bioassay! You see where we stand if we were to apply that to the human.

Fremont-Smith. We are in a spot because we are humans.

György And we are frustrated.

Holbrook· I think that is a good note on which to close.

REFERENCES

1 DAWSON, M H Comparative study of subcutaneous nodules in rheumatic fever and rheumatoid arthritis. *J. Exper. Med* **57,** *845 (1933)*

2 HOWES, E L Personal communication

3 JONES, I S, and MEYER, K Inhibition of vascularization of the rabbit cornea by local application of cortisone *Proc. Soc. Exper Biol & Med* **74,** *102 (1950).*

4 HUGHES, W F, JR Alkali burns of eye, review of literature and summary of present knowledge. *Arch. Ophth.* **35,** *423 (1946)*

5 CLARK, E R, and CLARK, E L: Microscopic observations on new formation of cartilage and bone in the living mammal *Am J Anat* **70,** *167 (1942)*

6 CLARK, E R Intercellular substance in relation to tissue growth *Ann New York Acad Sc* **46,** *733 (1946).*

7 INGLE, D J The relationship of diabetogenic effect of diethylstilbestrol to adrenal cortex in rat. *Am J. Physiol.* **138,** *577 (1943)*